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This paper is dedicated to Professor David Shugar on his 80th birthday

Human lipoprotein lipase deficiency: does chronic dyslipidemia lead to increased oxidative stress and mitochondrial DNA damage in blood cells?*

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Lipoprotein lipase (LPL) is a key enzyme in the metabolism of lipoproteins and their balanced distribution in the plasma. A deficiency of this enzyme due to gene mutations leads to severe dyslipidemia. In this report, we describe the major LPL gene mutations that are prevalent in the French-Canadian population of Québec and the nature of dyslipidemia caused by the resulting enzyme deficiency. We discuss the possibility that dyslipidemia caused by LPL deficiency may enhance oxidative stress in the blood cells, bring about increased fluidity of the membrane components of these cells and increase the susceptibility of their mitochondrial DNA to structural alterations. Some preliminary experimental results in verification of this hypothesis are presented.

Dyslipidemias represent heterogeneous pathological conditions in which the concentration, composition and distribution of lipids and lipoproteins in blood deviate considerably from the normal. Dyslipidemias have been classified into different phenotypes based on their plasma biochemical characteristics and associated clinical manifestations. Among the most important dyslipidemias present in the Québec population are the familial hypercholesterolemia (Fredrickson's type II dyslipidemia) and familial lipoprotein lipase (LPL) deficiency (type I dyslipidemia). Numerous genetic and epidemiologic studies have demonstrated that hypercholesterolemia poses a strong risk for the development of atheroscle-

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Abbreviations: Apo-B, apolipoprotein B; LPL, lipoprotein lipase; VLDL, LDL, IDL and HDL, very low, low, intermediate and high-density lipoproteins, respectively; OP, oxidative phospho- rylation; ROS, reactive oxygen species; Ter, translation termination.

rosis, leading to heart disease and stroke. Recent evidences indicate that triglyceride-rich lipoproteins, such as chylomicrons and very low-density lipoproteins (VLDL), may also represent important risk factors for coronary artery disease (for review see [1]).

Hydrogen peroxide and the oxygen free radicals (superoxide anion and hydroxyl), globally referred to as reactive oxygen species (ROS) are generated continuously in living cells from both enzymatic and nonenzymatic processes. Unless removed from the cellular environment, they react rapidly and indiscriminately with many of the critical biological molecules including proteins, nucleic acids and lipids, leading to their inactivation and producing secondary toxic products which further damage the cell. Although aerobic cells have evolved a number of mechanisms for reducing the levels of ROS and to limit oxidative stress [2], protection against ROS is not complete and, consequently, random structural alterations occur continuously throughout life, leading to chronic and irreversible age related pathologies. It has often been proposed that the cumulative and exponential effects of these changes are responsible for the increased likelihood of diseases with advancing age. Damage caused by ROS is implicated in a number of chronic and life-limiting diseases of humans, including atherosclerosis, cancer, arthritis and other degenerative diseases that accompany aging [3, 4].

Esterified unsaturated fatty acids in triglycerides and phospholipids of lipoproteins are the prime targets of oxidation by ROS. High plasma levels of such lipoproteins in a number of dyslipidemias may be expected to lead to their accumulation in the interstitial spaces of arterial intima, thus setting the stage for oxidation of these particles by ROS produced by the surrounding cells. Increased levels of plasma lipid peroxides have, indeed, been shown to occur in patients suffering from hypercholesterolemia and hypertriglyceridemia [5]. Of all blood lipids, low-density lipoprotein (LDL) is the most sensitive to oxidation [6, 7]. Two mechanisms, the generation of superoxide radicals and the endothelial release of lipooxygenase, are thought to be involved in the initiation and propagation of the autocatalytic chain reaction which results in the accumulation of oxidized LDL. The process of oxidation of LDL includes a series of sequential events including the loss of endogenous antioxidants, oxidation of long chain polyunsaturated fatty acids, formation of lysolecithin and modification of the ε-amino groups of lysine residues on apolipoprotein B (apo-B) molecules. These reactions reduce the net positive charge of apo-B and the resulting modified LDL is no longer able to be recognized by the normal LDL receptor. The uptake and metabolism of oxidized LDL occurs, on the other hand, by the scavenger pathway involving the monocyte/macrophage system and resulting in the formation of foam cells [1].

In addition to its role in the conversion of macrophages to foam cells, oxidized LDL is now known to exert more generalized effects on other phenomena such as cellular metabolism, cytotoxicity, inflammation, vascular tone, endothelial permeability and coagulation [8]. Cells in the S-phase of division appear to be more susceptible to the toxic effects of oxidized LDL and its products, probably because of the increase in bulk phase endocytosis in the S-phase or due to a disruption of the membrane processes essential for the cell cycle by the intercollation of oxidized lipids [9, 10]. DNA synthesis and γ-interferon production are also shown to be inhibited by oxidized LDL in concanavalin A-stimulated human monocytes [11].

There is now substantial evidence to indicate that oxidized LDL exists in significant amounts *in vivo*, under certain physio-pathological conditions and in small concentrations even in normal conditions (for a recent review see [12]). Atherosclerotic lesions, as well as plasma, are shown to contain antibodies specific for oxidized LDL. Oxidized LDL and lipid peroxidation products have also been detected in plasma of normal and diabetic rats and humans and there is strong evidence to support the belief that oxidized LDL occupies a crucial position in the development of atherogenesis.

The objectives of the present report are (a) to identify the major lesions of the LPL gene that are responsible for LPL deficiency in the French-Canadian population of Québec; (b) to demonstrate the effects of LPL deficiency on the relative distribution of plasma lipoproteins and on the membrane components of blood cells, and (c) to describe preliminary results designed to test the hypothesis that the structural integrity of the mitochondrial genome in blood

cells may become damaged by the abnormal lipoprotein composition of the ambient plasma.

NORMAL AND MUTANT FORMS OF LIPOPROTEIN LIPASE

Role of lipoprotein lipase in lipid metabolism

Chylomicrons, VLDL, LDL and high-density lipoproteins (HDL) constitute the four major classes of lipoproteins in human plasma [13]. They are discoid or spherical particles made up of a lipid component, consisting of different proportions of triacylglycerols, phospholipids, cholesterol and cholesterol esters, and a highly specific protein component consisting of one or more apolipoproteins. The chylomicrons and VLDL are mainly composed of triacylglycerol and are larger in size as compared to LDL and HDL which contain higher proportions of cholesterol and phospholipids. The chylomicrons are synthesized in the intestinal mucosa from dietary precursors and enter into circulation by way of the lymphatic system (Fig. 1). The VLDL particles are formed endogenously by hepatocytes. The nascent chylomicrons and VLDL are converted into their mature form by receiving apoproteins C and E from HDL one of whose important functions is to act as a reservoir and

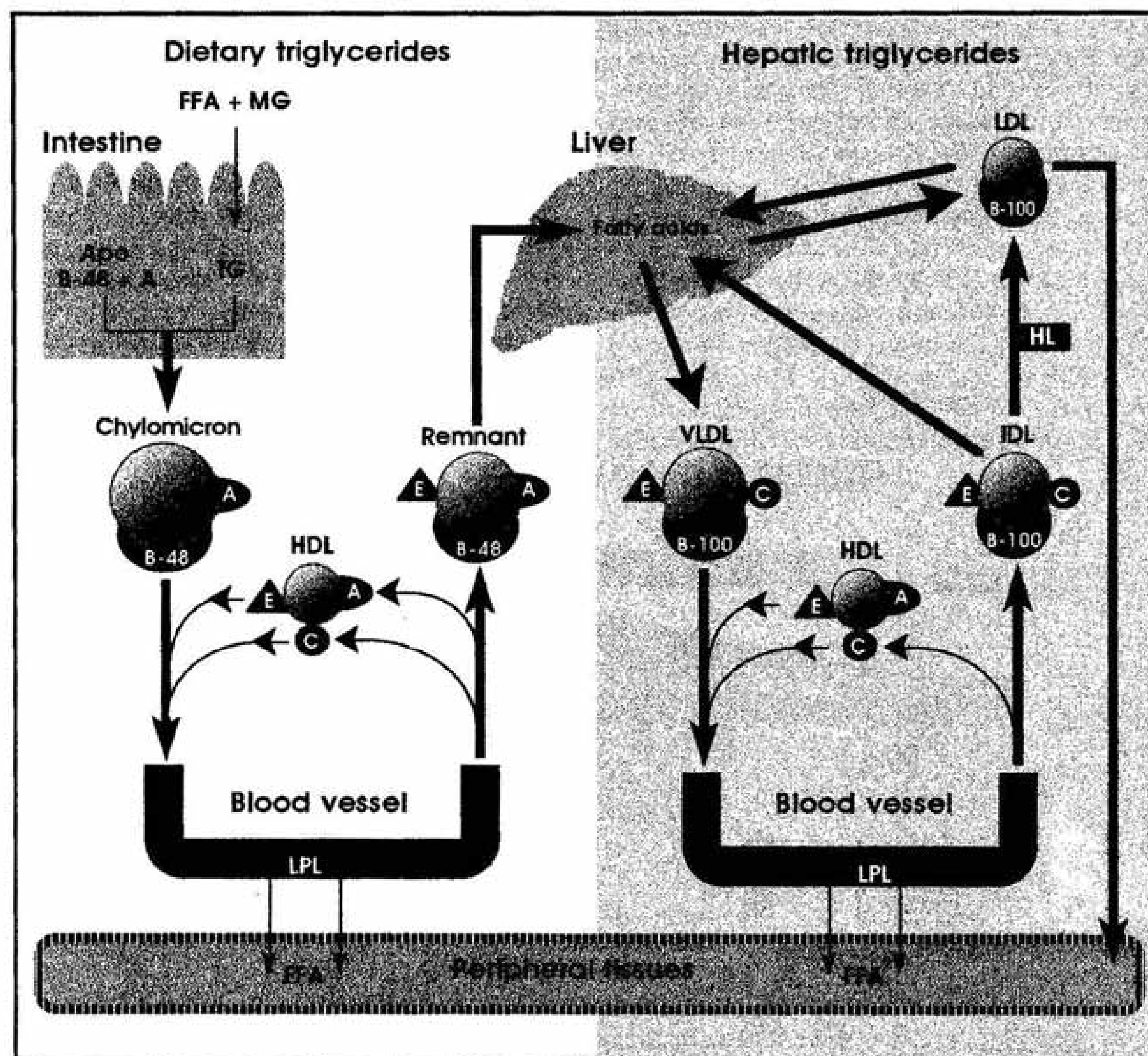

Fig. 1. Origin and metabolism of chylomicrons and very low-density lipoproteins (VLDL).
The various apolipoproteins are identified by A, B-48, B-100, C and E. FFA, free fatty acids; HDL, high-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; LPL, lipoprotein lipase; HL, hepatic lipase; MG, monoglycerides; TG, triglycerides.

donor of these apoproteins. Chylomicrons and VLDL are the primary substrates for the action of LPL.

The active LPL enzyme (EC 3.3.1.34) is a homodimer made up of two identical monomeric glycosylated polypeptides (for review see [14]). It is synthesized by parenchymal cells (adipose tissue, skeletal muscle, heart muscle, macrophages, etc.) and transported to its functionally active site, the luminal surface of capillary endothelial cells, where it is anchored by heparan sulfate to membrane glycoproteins. As the circulating chylomicrons and VLDL come into contact with LPL in these extrahepatic tissues, the triacylglycerol moiety is hydrolyzed and the fatty acids are taken up by the cells for storage (adipose tissue) or as a source of oxidative energy (muscle). The remnants of chylomicrons and intermediate-density lipoproteins (IDL) may undergo further exchanges of lipids and proteins with HDL and other plasma lipoproteins before they are finally picked up by cell receptors and catabolized by liver. An important alternate metabolic route for IDL is its conversion to LDL by the action of hepatic lipase. LDL is the unique donor of cholesterol for all tissues and this process is mediated by apo-B receptors on the surface of cells. Thus, LPL represents the first enzyme in the metabolism of both the alimentary and endogenous triglycerides and plays a key role in the formation and maturation of other plasma lipoproteins by providing precursors for synthesis as well as for exchange. For example, during the catabolism of chylomicrons and VLDL, HDL particles appear to bind more lipids and apoproteins and become converted to more buoyant forms of HDL [15]. Alterations in LPL activity, such as those caused by familial lipoprotein lipase deficiency, may therefore be expected to induce important dyslipoproteinemia.

Organization of the human LPL gene

The LPL gene is located in the p22 region of human chromosome 8 and is found in a single haploid copy (Fig. 2). It is approx. 30 kb in length and is divided into ten exons separated by nine introns [16, 17]. A number of regulatory elements including the tissue specific and hormone responsive sites have been identified in the 5′ region of the gene. All the amino acids of the LPL molecule are encoded by the first nine exons while the tenth exon harbors two polyadenylation sites, thus giving rise to two mRNA isoforms of different sizes (3.4 kb and 3.6 kb), but carrying the same coding sequence. In the maturation process, the signal peptide consisting of the N-terminal 27 amino acids is cleaved off and the remaining polypeptide of 448 amino acids is glycosylated.

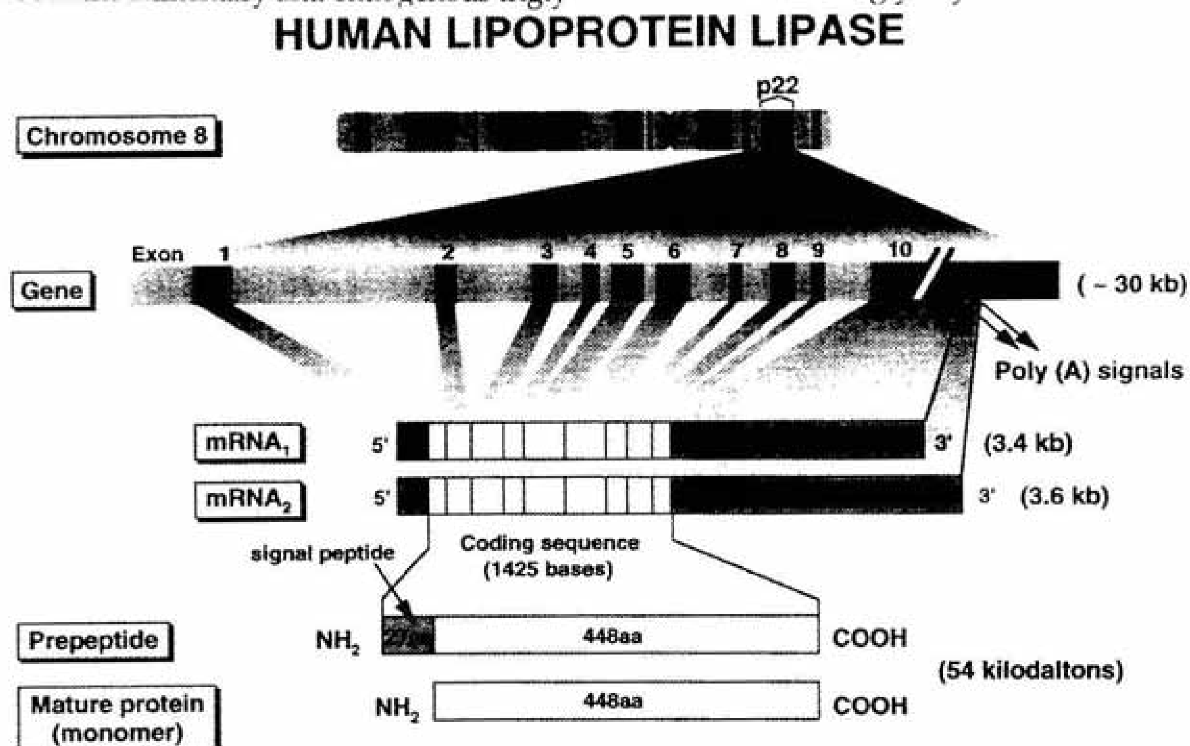

Fig. 2. Chromosomal location, organization and expression of the human LPL gene.

Mutations of the human LPL gene

More than seventy mutations have been identified in the human LPL gene [14] which include amino acid substitutions, premature termination, deletions, insertions and frameshift mutations (Fig. 3). The mutations appear to be distributed in a nonuniform fashion within the gene, being concentrated mostly in the middle exons. Certain codons are affected by more than one mutation, in different individuals. Most of the mutations lead to partial or complete loss of one or more functions of the LPL molecule.

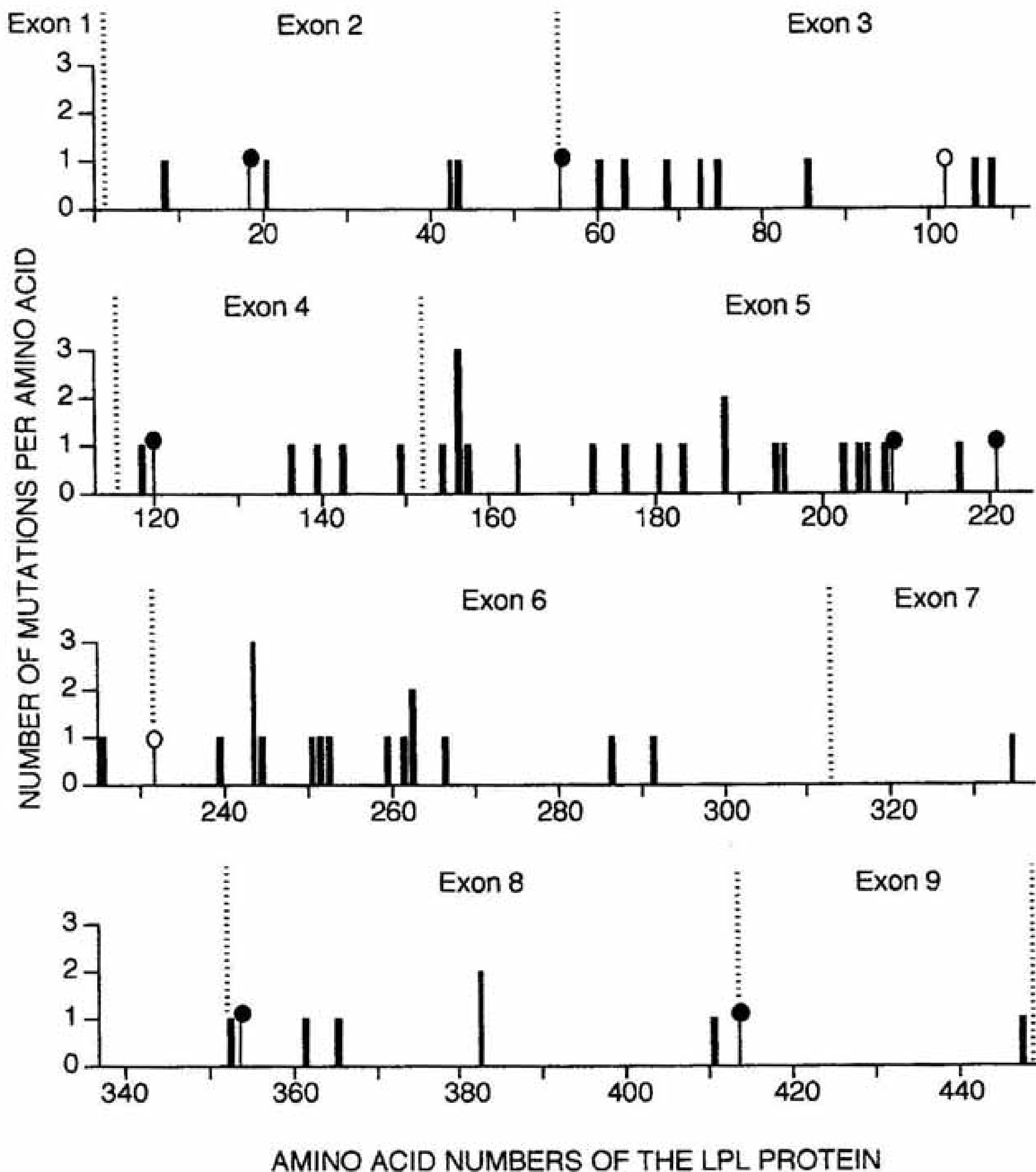

Fig. 3. Mutations found in the human LPL gene.
Data obtained from Murthy *et al.* [14]. The nine coding exons are demarcated by broken vertical lines. The amino acids affected by the mutations are shown by solid vertical lines whose length indicates the number of different mutations so far discovered (1 to 3 involving one or more of the three bases coding for that amino acid). Filled and open circles indicate base deletions or insertions.

LPL gene mutations prevalent in Québec

Five LPL gene defects have so far been identified in the French-Canadian population of Québec: Gly188Glu, Pro207Leu, Asp250Asn, Asn291Ser and Ser447, Ter. All these lead to complete loss of LPL catalytic activity in the postheparin plasma. Pro207Leu is the most common mutation in the region and accounts for 70% of the mutant alleles [18]. It is also almost exclusively French-Canadian and is mainly prevalent in the northeastern part of the province. Genealogical investigations have traced the origin of this mutation to a few founders who migrated to Québec from the northwestern part of France in the early 17th century [19]. Gly188Glu constitutes the next most common mutation with a distribution of approx. 25% of the affected alleles and is found mostly in Western Québec [20]. Although Québec has the highest concentration of this mutation, it is also found in other parts of the world and in different ethnic groups suggesting that it may represent a comparatively ancient mutation [21]. The other three mutations are present in small numbers in Québec and no genealogical studies have been made [14, 22].

Effects of LPL deficiency on plasma lipoproteins

LPL deficiency is generally recognized in homozygotes early in childhood by one or more clinical signs caused by the chylomicronemia, which include severe abdominal pain, splenomegaly, hepatomegaly, lipemia retinalis and eruptive xanthomas [23]. Pancreatitis is the major complication of chylomicronemia. Other likely symptoms are dyspnea, mono- or polyparesthesias and recent memory loss [24]. The fasting plasma of homozygotes appears lactescent due to accumulation of chylomicrons. The homozygous state may be further confirmed biochemically by the absence of LPL activity in the postheparin plasma and by gene analysis. The heterozygotes do not show chylomicronemia and are asymptomatic in regard to the usual clinical signs observed in homozygotes. For this reason, the heterozygous state often escapes detection except when suspected and verified by gene analysis, in families harboring a homozygote patient. Although LPL activity in the postheparin plasma of heterozygotes should theoretically be half that found in normolipidemic subjects, LPL activity measurements are not useful in individual diagnosis because of a large overlap in the normal and heterozygote values (Julien P. & Ven Murthy, M.R., unpublished).

Homozygotes have massive hypertriglyceridemia as a result of defective catabolism of chylomicrons and VLDL. Although both these lipoproteins are substrates of LPL, absence of this enzyme leads to highly elevated chylomicronemia, but does not affect either the number of VLDL particles or their composition in a majority of patients (Table 1). This is probably due to the fact that the level of VLDL in the plasma is the resultant of two mechanisms, its catabolism by LPL and its endogenous formation by liver. In the absence of LPL, it may be expected that the flow of fatty acids to liver is diminished, thus reducing VLDL synthesis. However, the reduced catabolism of VLDL in LPL deficiency is reflected in the reduced number of LDL particles. In heterozygotes, on the contrary, the number of VLDL particles are higher than normal, probably due to the partial presence of LPL. The differences in the lipoprotein profiles between the homozygote and heterozygote states could thus be due to the fact that the latter possesses significant LPL activity, although reduced by approximately half due to the mutant LPL allele.

The relative proportions of VLDL and LDL particles in the homozygotes and heterozygotes may thus be explained as a final consequence of the rates of VLDL catabolism and endogenous synthesis. LPL deficiency, both in homozygotes and heterozygotes, also leads to a reduced number of HDL particles and striking alterations in the relative proportions of lipids in the various lipoprotein particles (Table 1), affecting their size and density. The altered composition and sizes of lipoprotein may be attributed to the combined effects of LPL deficiency and the altered exchange of lipids and proteins between these various particles during their maturation and metabolism (Fig. 1). The sizes and densities of lipoproteins are important factors in their atherogenic potential. It is known that the oxidative susceptibility of LDL becomes amplified with increasing density, particularly in the dense LDL subfractions present in hypertriglyceridemic individuals [25]. There is experimental evidence suggesting that increased lipoprotein oxidation and associated lipoprotein atherogenicity

Table 1

Composition and characteristics of plasma lipoproteins in LPL deficiency[a]

Homozygotes	
Plasma:	Severe hypertriglyceridemia and increased cholesterol level
Chylomicrons:	Very elevated
VLDL:	Normal number of particles Normal lipid composition in the majority of patients
LDL:	Reduced number of particles Cholesterol-poor particles (denser particles based on cholesterol/apo-B ratio)
HDL:	Reduced number of particles Cholesterol-poor particles
Heterozygotes	
Plasma:	Moderate increase in triglyceride levels
Chylomicrons:	Absent
VLDL:	Increased number of particles Increased triglyceride and cholesterol levels (more buoyant particles)
LDL:	Normal number of particles Cholesterol-poor particles (denser particles based on cholesterol/apo-B ratio)
HDL:	Reduced number of particles Cholesterol-poor particles

[a]Data obtained from Cantin *et al.* [43], Julien *et al.* [27, 50], Sniderman *et al.* [29] and Murthy *et al.* [14].

could be a common characteristic in hyperlipidemia [26]. Although the heterozygous state of LPL deficiency does not exhibit striking clinical symptoms associated with the total absence of LPL activity, the abnormal composition, numbers, sizes and densities of plasma lipoprotein particles suggest that it may represent a form of familial dyslipoproteinemia with a significant atherogenic risk [14, 27–29].

VULNERABILITY OF THE MITOCHONDRIAL GENOME TO OXIDATIVE STRESS

Mitochondrial DNA (mtDNA)

Mitochondria house the entire pathway for the tricarboxylic cycle, respiratory chain and oxidative phosphorylation, contain many of the key enzymes for the synthesis of fatty acids, phospholipids, ketone bodies and for gluconeogenesis, and share the major part of β-oxidation of fatty acids with peroxisomes. mtDNA constitutes approx. 0.3% of the total human genome. It is a double stranded circular molecule of 16569 bp and codes for 13 protein subunits of the oxidative phosphorylation (OP) complex, 2 rRNAs and 22 tRNAs. The rest of the subunits of the OP complex and other mitochondrial proteins are encoded by nuclear DNA (nDNA). The molecular biology of mtDNA differs from that of nDNA in several respects. For example, the genetic information in mtDNA is very closely packed; both the heavy and light strands are transcribed and there are very few noncoding sequences. Mitochondrial gene expression follows a prokaryotic system inside an otherwise eukaryotic cellular environment. The genetic code of mtDNA is also different from that of nDNA in regard to the number of tRNAs required for protein synthesis, the codons used for certain amino acids and for termination of translation (for review see [30]). In addition, the genetics of mtDNA has special features that have important implications for human disease. Since each cell contains hundreds of mitochondria and many more copies of mtDNA, mixtures of normal and mutant mtDNAs may coexist in a single cell. Unlike nDNA, mtDNA is inherited largely through the maternal line and replicated independently of the nDNA. During cell division, the mtDNA molecules are randomly

distributed to the daughter cells by replicative segregation.

Generation of ROS and oxidative stress in mitochondria

A major source of ROS in the aerobic cell is the leakage of electrons on to molecular oxygen from various components of the electron transport chain in mitochondria. Superoxide and hydrogen peroxide formation normally accounts for 1–2% of mitochondrial oxygen consumption when respiratory chain carriers located on the inner mitochondrial membrane are highly reduced [31, 32]. Mitochondrial regions comprising ubiquinone-cytochrome *b*, NADH dehydrogenase and dihydroorotate dehydrogenase are major contributors to superoxide production [32]. Hydroxyl radicals which are the most reactive of the three partially reduced oxygen intermediates [33] are generated from hydrogen peroxide and superoxide in the presence of transition metals such as iron [34]. Iron released during the turnover and degradation of heme containing cytochrome complexes of the respiratory chain may accelerate this process. Because of their high reactivity and short life, the hydroxyl radicals produced in mitochondria would be expected to produce deleterious effects mainly in this organelle [35].

Mitochondrial membranes as targets of ROS

Mitochondria consist of two membranes, an outer membrane and a highly invaginated inner membrane. The integrity of both the inner and the outer membranes is essential for mitochondrial function, since they are responsible for an orderly passage of biological molecules, for segregating them into appropriate compartments and for protection of the mitochondrial machinery against endogenous and exogenous toxins. The outer membrane possesses relatively few enzymatic and transport functions while the inner membrane contains most of the enzymes required for electron transport and oxidative phosphorylation. Several dehydrogenases and a number of transport systems for carrying metabolites between the cytosol and the mitochondrial matrix are also present in the inner membrane. The association of these various components with the membranes vary in tightness and in their susceptibility to physical and chemical agents. In addition to proteins which are modified by ROS [36], both membranes contain a high proportion of phospholipids and polyunsaturated fatty acids [37] which are the most frequent substrates for free radical peroxidation. Lipid peroxides may then be degraded to cytotoxic aldehydes and hydrocarbons in the presence of iron originating from the mitochondrial cytochromes. All of this could have the effect of causing membrane disruption or increased membrane fluidity, thus exposing the mtDNA to the action of ROS and other disruptive agents. The topic of lipid peroxidation in mitochondria has recently been reviewed by Bindoli [38].

mtDNA mutations and human pathology

DNA is a major target of ROS, their direct action resulting in base substitutions, strand breaks and deletions [39]. The spontaneous mutation rate of mtDNA is considerably higher than that of nDNA. This is further compounded by the lack of efficient mechanisms in mitochondria for DNA protection and repair such as those present in the nucleus. For example, mtDNA are not covered by histones and there is no significant recombinational repair in this organelle, with the result that the steady state level of oxidized bases in mtDNA is about sixteen times as high as in nDNA [40]. However, certain types of mtDNA repair, such as the excision of modified bases, may be possible, since mitochondria contain uracil DNA glycosylases, apurinic/apyrimidinic endonucleases and UV endonucleases [41]. It is not known, however, whether these enzymes are involved in the repair of damaged DNA or in the degradation of the damaged DNA.

A variety of chronic degenerative diseases that affect the brain, heart, muscle, kidney and endocrine glands have been shown to result from mutations in mtDNA. Among these are LHON (Leber hereditary optic neuropathy) which involves mutations in the subunits 1 and 4 of NADH dehydrogenase, maternally inherited mitochondrial neuropathy resulting from mutation in ATPase 6, MERRF (myoclonic epilepsy with ragged red fibers), MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) and fatal infantile cardiomyopathy produced by mutations in $tRNA^{Lys}$ (TjCG loop), $tRNA^{Leu}$ (DHU loop) and $tRNA^{Ile}$ (TjCG stem), respectively. The unorthodox genetics, late onset and pro-

gression of these diseases suggest that a variety of more common degenerative disorders (including heart disease, adult-onset Alzheimer's disease and aging) may also be related to mtDNA mutations (for reviews see [3, 4]).

DOES DYSLIPIDEMIA RENDER mtDNA OF BLOOD CELLS VULNERABLE TO OXIDATIVE STRESS?

In the course of our studies on the effects of LPL deficiency on lipid metabolism, we have observed an increased tendency for *in vitro* hemolysis of erythrocytes from homozygote LPL deficient patients, even at physiological osmotic conditions, indicating an increased membrane fragility [42]. This phenomenon became more marked as the osmotic force was increased to 150 mOsm or more. This has led us to investigate the effects of LPL deficiency on erythrocyte lipid composition and membrane fluidity in these patients. Anisotropy, which is directly proportional to membrane microviscosity and inversely proportional to membrane fluidity, was determined by fluorescence polarization measurements of erythrocyte membranes. Our results show that LPL deficiency leads to increased fluidity of the membranes (Fig. 4). This is accompanied by a significant deviation in the composition of membrane lipids, particularly in cholesterol content (mol/mg membrane protein) which was decreased, as compared to controls. While the concentration of total phospholipids remained unchanged, the relative distribution of different classes of phospholipids was altered. For example, lysophosphatidylcholine and sphingomyelin were decreased while phosphatidylcholine was increased. The relative concentrations of phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine were not significantly affected. We have also observed changes in the lipid profiles of homozygote LPL deficient plasma [42, 43] suggesting that these complex events are probably produced, at least in part, by exchanges of lipoprotein constituents, particularly cholesterol and phospholipids, between the plasma and the membranes of circulating cells, in the dyslipidemic environment caused by LPL deficiency.

The generation of ROS both within and outside mitochondria, the enhanced potential for oxidative stress and the accumulation of oxidized lipids in conditions of hyperlipidemia, suggest that mtDNA may be especially vulnerable to chronic dyslipidemia caused by LPL deficiency or by secondary pathologies associated with deficiency in LPL activity. mtDNA is attached, at least transiently, to the inner mitochondrial membrane where large amounts of

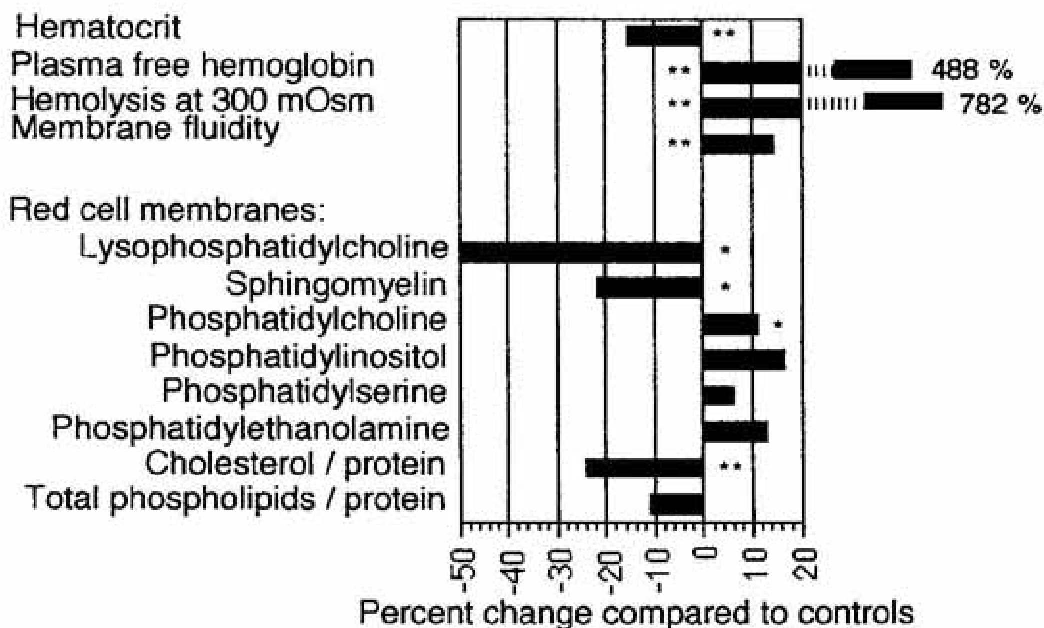

Fig. 4. Osmotic fragility and membrane lipid composition of erythrocytes in human LPL deficiency.
The asterisk indicates values that are statistically significant: * $P < 0.05$ and ** $P < 0.005$

ROS are produced. There is experimental evidence that ROS may bring about structural damages in mtDNA indirectly by lipid peroxidation of mitochondrial membranes [44, 45]. This situation may be aggravated by possible alterations in the structure and composition of the mitochondrial membranes due to the abnormal lipid composition of the ambient milieu as discussed above for erythrocytes. Since mitochondria play a major role in the synthesis and oxidation of fatty acids, any mutations in the mtDNA may adversely affect fatty acid metabolism and contribute to a further deterioration of the dyslipidemic conditions.

As a first step in testing the hypothesis that LPL deficiency may affect the integrity of mtDNA, we have investigated the occurrence of major structural alterations (large deletions or insertions) in mtDNAs isolated from blood lymphocytes of LPL deficient patients and compared them to other types of dyslipidemia and to normolipidic controls. Blood cells represent the most accessible and most readily available of human tissues. They exist in continuous and intimate contact with the dyslipidemic plasma and their mtDNA would therefore be expected to show any damage resulting from the abnormal environment in their milieu. Reasons for the selection of lymphocytes are related, among other factors, to their abundance, mitochondrial content and certain special features of their metabolism [46]. Lymphocytes originate from lymphopoietic stem cells in fetal liver and mature into several functional types, the major classes being the T and B cells. In normal adults, there are about 2.5×10^9 lymphocytes per liter of blood which amounts to nearly 50% of the total leukocyte population. A great majority of lymphocytes are long lived ranging from a few months up to five years. They possess mitochondria which produce most of the energy required for lymphocyte locomotion. Lymphocytes are particularly vulnerable to physical and chemical agents that degrade DNA, since (a) they are rich in endonucleases; (b) they have only small pools of deoxynucleotide triphosphates that limit the rate of DNA repair and (c) in contrast to other leukocytes (neutrophils, eosinophils and monocytes), they do not contain peroxidase activity, thus rendering them susceptible to peroxides generated in mitochondria and elsewhere in the cell.

The survey of deletions and insertions in mtDNA was carried out by PCR amplification of consecutive overlapping regions of mtDNA and examination of the amplified products for the presence of abnormal bands. The preparation of mtDNA and the oligonucleotide primers used are described in Table 2. In order to optimize PCR conditions for each of the primer pairs, a large number of different variants were tested including the temperatures and durations of annealing, polymerization and denaturation, hot-start procedures, relative concentrations of primers, template DNA and ions, different sources and levels of Taq polymerase, number of polymerization cycles etc.

PCR amplification of DNA, in the presence of a pair of specific primers, is expected to produce a product defined ideally by a single band on gel electrophoresis. In theory, the appearance of other larger or smaller bands, in addition to the main band, may suggest insertions or deletions within the target region flanked by the two primers. However, in practice, these secondary bands may also arise as a result of partial homology between some other regions of the template DNA and the 3′ growing ends of the primers. In extreme situations where annealing stringency and other PCR conditions are not previously optimized for the DNA template and the primers, these secondary bands may become preferentially amplified to the detriment of the target site, particularly if they are smaller in size. We have discussed these and other problems associated with PCR analysis in detail elsewhere [47]. Although these difficulties can generally be resolved by optimization of individual reaction conditions, by the use of internal primers, by restriction fragment analysis and eventually by sequencing of the DNA bands, they pose a situation of ambiguity when it is intended to survey large numbers of target DNAs for preliminary identification of defective molecules. However, in the case of mtDNA which is a short circular molecule whose entire sequence is known, the primers and the DNA can be compared for regions of homology and those secondary bands resulting from misannealing may be predicted and identified. Such a homology analysis was carried out and a detailed list of main and secondary bands was compiled. Any amplification product which did not correspond in size to the main band or any of the predicted secondary bands was pro-

Table 2
Oligonucleotide primers used for PCR amplification of human mitochondrial DNA[a]

L-strand primer	Sequence position on L-strand	H-strand primer	Sequence position on L-strand
5′-GATCACAGGTCTAT CACCCT-3′	1–20	5′-TATTATTATGTCCTA CAAGC-3′	240–221
5′-CAGACGAGCTACCT AAGAAC-3′	1911–1930	5′-TGGTTTTCGGGGGT CTTAGCT-3′	1912–1893
5′-TCTAGCCACCTCTA GCCTAG-3′	3621–3′640	5′-ATGCTACCTTTGCA CGGTTA-3′	2599–2580
5′-AGTCCCAGAGGTTA CCCAAG-3′	4811–4830	5′-CGTTCGGTAAGCAT TAGGAA-3′	3380–3′361
5′-TACCCATCATAATC GGAGGC-3′	6115′–6134	5′-CTAGGCTAGAGGT GGCTAGA-3′	3640–3′621
5′-TTCATGCCCATCGT CCTAGA-3′	8201–8220	5′-TGATGGCAGGAGT AATCAGA-3′	3840–3′821
5′-CCCTCTACCCCCTC TAGAGCCCACTGTAA AGC-3′	8274–8305	5′-AGTAGGGTCTTGGT GACAAA-3′	4100–4081
5′-ACGAAAATCTGTTC GCTTCA-3′	8531–8550	5′-TCATAGTCCTAGAA ATAAGG-3′	4340–4321
5′-TCTAAGATTAAAAA TGCCCT-3′	8881–8900	5′-GCTAGCATGTTTAT TTCTAG-3′	4585′–4566
5′-ACACTAACCATATA CCAATG-3′	9357–9376	5′-CTTGGGTAACCTCT GGGACT-3′	4830–4811
5′-CCTTCACCATTTCC GACGGC-3′	9759–9779	5′-TGATGCCTCCTATG ATGGCA-3′	7083–7064
5′-CTACCATGAGCCCT ACAAAC-3′	10281–10300	5′-GGATCAATAGAGGG GGAAAT-3′	8620–8601
5′-CTCACAAGAACTGC TAACTC-3′	12214–12233	5′-AGGGCATTTTTTAA TCTTAGA-3′	8900–8881
5′-CGGATCATTTCTCTA CTCAG-3′	15058–15077	5′-GATGACATAACTAT TAGTGG-3′	10330–10311
5′-GGAGGACAACCAG T AAGCTA-3′	15761–15780	5′-TCTTGTTCATTGTTA AGGTT-3′	13400–13381
5′-GCCAGCCACCATGA ATATTG-3′	16106–16125	5′-GGCTTCCGGCTGC CAGGCCTTTAATGGG-3′	13720–13693
		5′-GTAGGCGAATAGG AAATATC-3′	5580–15561

[a]L-strand and H-strand primers are those with sequences identical to, and complementary to, the L-strand of the mtDNA, respectively [51].

visionally considered an abnormal band and was presumed to arise from a structural alteration within the targeted DNA site. Abnormal bands which appeared consistently under severe stringent PCR conditions were then selected for further analysis with the view of precisely identifying the location and the nature of the lesion within the molecule.

Abnormal bands were found in lymphocyte mtDNAs of all types of dyslipidemic patients examined as well as in control subjects (Table 3). No bands characteristic of a particu-

Table 3
Detection of abnormal bands following PCR of human mitochondrial DNA

Disease category	Number of subjects	Number with abnormal bands
Controls	9	9
Homozygotes for LPL deficiency	3	3
Heterozygotes for LPL deficiency	6	6
Homozygotes for familial hypercholesterolemia	4	4
Heterozygotes for familial hypercholesterolemia	7	6
Familial combined hyperlipidemia	4	4
Hypertriglyceridemia	5	4

lar disease or common to a group of diseases were identified. The abnormal bands were not clustered in any particular region of mtDNA, but appeared to originate from different parts of the molecule indicating that, if these bands did indeed represent real structural alterations in mtDNA, they must have occurred in a non-selective random fashion. We are, at present, carrying out further experiments, including the quantitation and sequencing of some of these abnormal bands, in order to establish whether there exist any proportional or qualitative differences among the various groups and between the dyslipidemic and control subjects.

LPL deficient patients are normally placed under dietary and medical surveillance as soon as the diagnosis of LPL deficiency is confirmed, with the result that the degree of exposure of blood cells to chronic dyslipidemia is often ambiguous or unknown. In an attempt to obviate this difficulty, we are at present proposing to use monocyte-macrophage cells cultured *in vitro* under defined conditions, in the presence of various combinations of lipoproteins, oxidants and antioxidants. Recently, we have developed methods for amplification of very large segments of mtDNA using purified mtDNA from a small number of cells [48, 49]. This will permit scanning of the entire mitochondrial genome using a minimum number of primers, thus avoiding many of the PCR related artifacts. At the same time, we have also been examining structural alterations of mtDNA at a finer level (base substitutions, base modifications, strand breaks, conformational changes etc.) which could be caused by dyslipidemia and which could have equally deleterious effects on mitochondrial function.

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