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HDL: A molecular view of the «classic» antiatherogenic lipoprotein

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Abstract

HDL metabolism and its relationship to atherosclerosis is a complex topic, though notable advances have been made. First, HDL clearly has an anti-atherogenic action. Second, this action is due to some fraction/s containing apoA-I but not apoA-II. Third, some of the molecular mechanisms involved in two key anti-atherogenic functions of HDL have been defined: reverse cholesterol transport and prevention of LDL oxidative modification. These advances may permit the development of effective strategies, complementing those already available, for treating and preventing atherothrombotic cardiovascular diseases.

Resum

L'estudi del metabolisme de les HDL (lipoproteïnes de densitat alta) i la seva relació amb l'arteriosclerosi és un tema complex, en el qual, recentment, s'han fet avenços importants. Primer, s'ha demostrat que les HDL tenen una acció antiaterogènica. Segon, s'ha establert que aquesta acció és deguda a fraccions que tenen apoA-I però no apoA-II. Tercer, s'han definit alguns mecanismes clau en l'acció antiaterogènica de les HDL: transport invers de colesterol i prevenció de la modificació oxidativa de les LDL (lipoproteïnes de densitat baixa). És previsible que aquests avenços permetran el desenvolupament d'estratègies efectives, que complementaran les existents per al tractament i la prevenció de les malalties cardiovasculars aterotrombòtiques.

Keywords: High-density lipoprotein, atherosclerosis, hypoalphalipoproteinaemia, apolipoprotein, cardiovascular disease.

1. Introduction

High-density lipoproteins (HDL) form one of the main lipoprotein families present in plasma. It is a lipoprotein family of very heterogeneous particle size, apolipoprotein composition and density (which varies between 1.063 g/ml and 1.21 g/ml). Cholesterol transported by HDL is a protective factor against atherosclerosis, whereas the cholesterol present in low-density lipoproteins (LDL) constitutes a risk factor. It is therefore generally considered positive to have a high concentration of HDL cholesterol in plasma together with a low level of LDL cholesterol. These concepts have their basis in several clinical and epidemiological studies that have demonstrated an inverse relationship between the concentration of plasma HDL cholesterol and the risk of suffering atherosclerosis, while this relationship is direct in the case of LDL cholesterol [1, 2]. Nevertheless, the relationship between HDL and atherosclerosis is complex and has exceptions. For example, patients with very infrequent genetic alterations which cause almost total deficiencies of HDL do not appear to run any greater risk of atherothrombotic cardiovascular disease than control subjects [3]. This feature has recently been reproduced in animals in which genes of vital HDL proteins were specifically knocked out [4]. As an explanation for these discrepancies, it has been postulated that the relationship between the concentration of HDL cholesterol and atherothrombotic cardiovascular disease might only be secondary to alterations in the metabolism of triglyceride-rich lipoproteins [3]. However, the antiatherogenic capacity of increased HDL plasma concentration has recently been demonstrated in animal models [5-10]. Another explanation for the lack of increased cardiovascular risk of many HDL deficiencies is that these usually present with a concomitant considerable reduction in LDL cholesterol [11].

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The functions of HDL are also controversial, especially those which are probably determinant in their antiatherogenic action. This review summarises the present vision of the relationship between HDL and atherosclerosis, particularly concerning its molecular bases.

2. Structural characteristics and HDL classification

The name «HDL» implies the use of a criterion of density to classify lipoprotein families. The various classifications and terminologies (which depend on the method used) for HDL are shown in Table 1. They are particularly relevant given that many of the studies on HDL attempt to assign the antiatherogenic action of HDL to specific particle subpopulations. Using this same criterion of density as a classification, two predominant HDL types, HDL2 (density between 1.063 g/ml and 1.125 g/ml) and HDL3 (density between 1.125 g/ml and 1.210 g/ml), can be distinguished. Although it is a very minor fraction in humans, HDL1 or apolipoprotein E (apo E)-enriched HDL is also found in other animals. This fraction is less dense than HDL2, although its limits are not well defined. Its high apoE content aids its characterisation. Another form of classification, of growing importance, classifies HDL particles according to their apolipoprotein content. ApoA-I is quantitatively the most abundant protein in HDL, followed by apoA-II. This gives rise to the frequently used concepts of HDL with apoA-I and without apoA-II, HDL with apoA-I and with apoA-II, and HDL with apoA-II and without apoA-I [12]. This classification does not include the content of HDL apolipoproteins: C-I, C-II, C-III, A-IV, E, D, J and H. Thus, even within each of these types of particles, there is marked apolipoprotein heterogeneity. For a summary of the characteristics of HDL apolipoproteins, see Table 2. Another classification is based on the electrophoretic mobility in agarose gels of HDL populations, according to their electric charge. These are classified as α, pre-β and γ. HDL can be separated for size by non-denaturing gel electrophoresis in polyacrylamide gradient gels. The populations obtained with this method, which are similar to those obtained by ultracentrifugation, are called (from smaller to greater size) HDL3c (7.62mm), HDL3b, HDL3a, HDL2a and HDL2b (10.57 mm) [13].

Table 1. HDL classification depending on the methodology used for their isolation.

Ultracentrifugation	HDL_2 (density between 1.063-1.125 g/ml) HDL_3 (density between 1.126-1.210 g/ml)
Electrophoresis in polyacrylamide gels	HDL_{2b} (particle diameter, 10.6 nm) HDL_{2a} (particle diameter, 9.2 nm) HDL_{3a} (particle diameter, 8.4 nm) HDL_{3b} (particle diameter, 8 nm) HDL_{3c} (particle diameter, 7.6 nm)
Electrophoresis in agarose gels	α-HDL preβ-HDL γ-HDL
Immunoaffinity chromatography	HDL with apoA-I and without apoA-II HDL with apoA-I and apoA-II HDL without apoA-I and apoA-II

Table 2. Characteristics of HDL apolipoproteins.

Name	*Molecular weight (kD)*	*Plasma concentration (mg/dl)*	*Function*
apoA-I	28	130	structural, LCAT cofactor, cholesterol efflux, SRB-I ligand
apoA-II	17	40	?
apoA-IV	46	12	LCAT cofactor
apoC-I	6.5	12	favouring triglyceride exchange
apoC-II	8.8	12	LPL cofactor
apoC-III	8.7	12	LPL inhibitor
apoD	29	5	transport of lipophilic substances?
apoE	34	2	Ligand for receptors, cholesterol efflux
apoJ	70	?	clearance of apoptotic residues?
apoH	43	20	inhibition of coagulation?
apoK-45	45	4	inhibition LDL oxidative modification, Hydrolysis of homocysteine thiolactone

3. ApoA-I

3.1. Structure and properties

ApoA-I is the principal protein component (68%) of HDL. It is also present in small quantities in chylomicrons, but not in lipoproteins which contain apoB-100. Thus, this apolipoprotein is only found in physiological conditions in HDL during fasting, when clinical analyses are usually made. ApoA-I is made up of 243 amino acids and has an approximate molecular weight of 28 kD [14]. It is synthesised in liver and intestine, as a product of a gene found in the q23 region of chromosome 11, near the apo C-III/apoA-IV/insulin gene cluster [15]. The apoA-I gene belongs to a family of genes which codify small and soluble apolipoproteins (A-I, A-II, A-IV, E, C-I, C-II and C-III), characterised by a structure of 4 exons and 3 introns. Exon 4 codifies many repeated series of 11 or 22 amino acids that generate zones of the α-helical properties called amphipathic helices (Figure 1). These, on interacting with phospholipids, are formed by two opposing sides, one hydrophobic and the other hydrophilic. The hydrophobic side is in contact with the aliphatic chains of fatty acids, while the hydrophilic part is in contact with the aqueous medium, interacting with the most polar region of phospholipids and with free cholesterol [16].

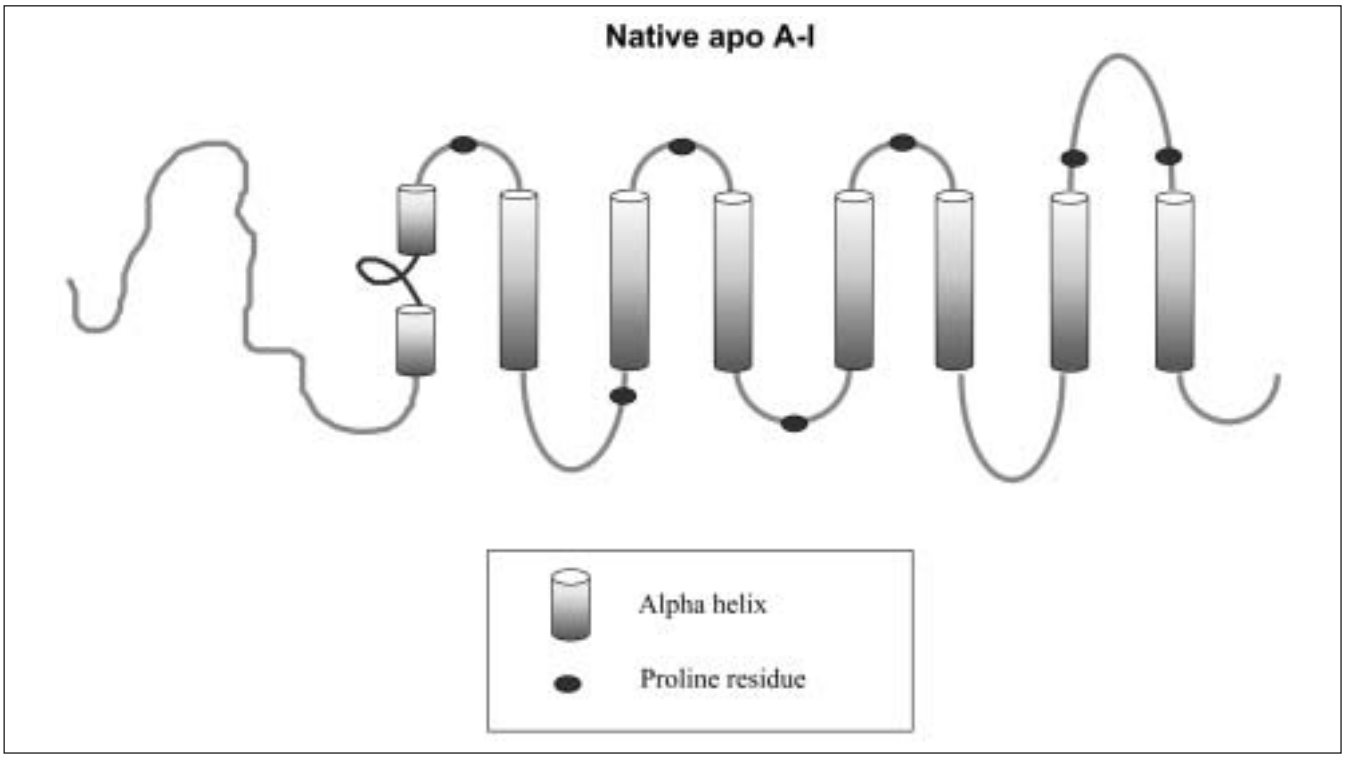

Figure 1. A structural model of apolipoprotein A-I, common to other soluble apolipoproteins, in which the amphyphatic helices are separated by prolines.

After its synthesis, apoA-I is called pre-pro-apoA-I, which is a precursor form of high molecular weight. Upon passing to cytoplasm, it is converted into pro-apoA-I. Subsequently, in the bloodstream and lymph, it is converted into the mature protein through the action of an as yet not well characterized plasmatic converter enzyme [17].

ApoA-I has an important structural function in HDL, since its deficiency is associated with HDL deficiency. Various models of apoA-I structure in nascent discoidal HDL have been proposed [18]. In one of the two more credible models, the helices are parallel to the plane of the lipid disc and, in the other, the helices are anti-parallel [18]. The physicochemical characteristics of the two models make the parallel structure seem more likely, at least in a model of association of up to two molecules of apoA-I in a single lipid disc [18]. The spheroidal particles of HDL contain from 2 to 4 molecules of apoA-I whose structure is probably similar to that of discoidal HDL [18]. ApoA-I plays a fundamental role in the first steps of the efflux of cholesterol from the cells (see section 8.1.1 for more details) and is the cofactor of the enzyme lecithin:cholesterol acyltransferase (LCAT) (19).

3.2. Mutations of the apoA-I gene

The hypothesis that HDL deficiencies caused by apoA-I deficits increase the risk of atherosclerosis accounts for the effort made to characterise mutations of the apoA-I gene and establish the genotype-phenotype associations of the different mutations found. Although, at first, some patients with deletions and inversions of the apoA-I/apoC-III/apoA-IV cluster seemed to present the expected increase in coronary atherosclerosis, after the description of more than 20 mutations of apoA-I (although not all affect the concentration of HDL cholesterol) this hypothesis has not been confirmed [for a thorough review, see references 3 and 20]. Neither patients with almost total HDL deficiency nor their family members present a greater risk of atherosclerosis than the control population [11]. Three of these apoA-I mutations which greatly diminish HDL cholesterol but are not associated with a greater risk of atherosclerosis are Arg173Cys (apoA-I Milano), Pro165Arg, and Gly26Arg (apoA-I Iowa) [3]. Surprisingly, apoA-I Milano seems to be an important protective factor against atherosclerotic cardiovascular disease. In support of this, epidemiological as well as experimental data demonstrate that its administration inhibits fattening of the carotid intima in hypercholesterolaemic rabbits and in apoE knock-out mice, which are models of accelerated and massive atherosclerosis, and that it inhibits thrombosis [21-23]. Several apoA-I mutations have been found in patients with amyloidosis (for example, Gly26Arg, Leu60Arg and deletion of residues 60-71 of the protein which were substituted by valine and threonine), although the nature of this association is unclear [24]. Another interesting case is that of the mutations of apoA-I which cause dominant autosomal inherited hypoalphalipoproteinaemia. These are apoA-I Milano (Arg173Cys), apoA-I Seattle (deletion of amino acids 146-160), apoA-I Paris (Arg151Cys), apoA-I Finland (Leu159Arg) and apoA-I Oslo (Arg160Leu) [25]. We recently studied various generations of one family from the Mediterranean island of Mallorca, who have a history of dominantly inherited hypoalphalipoproteinaemia due to a heterozygous deletion of amino acids 165-175 of the apoA-I (apoA-I Mallorca) (Martín-Campos *et al.*, J. Lipid Res. 2002;

43:115-123). This family does not show any increased cardiovascular risk, even though the proband was studied because of coronary atherosclerosis. In all apoA-I Mallorca carriers there is failure to activate LCAT, suggesting that the presence of a molecule of mutated apoA-I could alter the capacity of cholesterol esterification in HDL [25] and cause an increase in HDL, as has been described in inherited forms of primary LCAT deficiency.

3.3. ApoA-I transgenic animals

The first generated human apoA-I transgenic mice expressed the transgene only in liver, despite the fact that the microinjected construction contained 5.5 Kb of the 5' extreme and 4 Kb of the 3' extreme. Hence, it was concluded that the genetic elements, which control apoA-I expression in the intestine, differ from the liver elements and are outside the above-mentioned genomic region [26]. A later study demonstrated that the site in which intestinal expression of apoA-I is induced is found in the DNA situated between 0.3 Kb and 1.4 Kb of the 5' extreme of the gene of the adjacent apoC-III [27]. In fact, it is suspected that this region also controls intestinal expression of the other two components of the cluster, the apoC-III and apoA-IV genes.

Human apoA-I transgenic mice have higher HDL cholesterol than controls, which correlates with the concentration of human apoA-I. These transgenic mice are deficient in mouse apoA-I through an undetermined post-transcriptional mechanism. Overexpression of human apoA-I in the mouse reproduces the size heterogeneity characteristic of human HDL as well as the increase in concentration of apoA-I and HDL cholesterol produced in response to fat-rich diets [28-30]. This contrasts with characteristics of control mice in which HDL size is homogeneous and in which a fat-rich diet decreases the plasma concentration of apoA-I and HDL cholesterol. Thus, differences between the structure of human and mouse apoA-I determine the size profile of the HDL particles as well as their plasma concentration in response to a fat-rich diet. The generation of an animal model in which HDL increases after a fat-rich diet is relevant because it permits detailed study of the molecular mechanisms governing the plasma concentration of apoA-I and HDL cholesterol in pro-atherogenic conditions [31].

The development of transgenic mice, rats and rabbits for the apoA-I gene has permitted study of their effect on atherosclerosis susceptibility. The conclusion of the different studies appears clear: apoA-I overexpression prevents atherosclerosis in mice and rabbits fed a fat-rich diet [7, 32]. Overexpression of human apoA-I also prevents accelerated atherosclerosis after angioplasty in rats, apparently by reduction in the proliferation of muscle cells [33]. Additionally, overexpression of apoA-I in transgenic mice prevents atherosclerosis in genetically-modified mice which suffer massive atherosclerosis. This is the case of apoE-deficient mice [9, 10] and apo(a) transgenic mice [8]. In conclusion, these studies demonstrate that apoA-I overexpression leads to an increase in HDL cholesterol and is able to prevent atherosclerosis. This concept coincides with epidemiological data which found an inverse relationship between HDL cholesterol and the risk of coronary atherosclerosis.

Genetically-modified mice by inactivation (knock-out) of the apoA-I gene have also been produced [4, 34]. These animals are deficient in HDL, thereby demonstrating that apoA-I is critical for HDL structure [4]. The absence of apoA-I causes LCAT functional deficiency, which also illustrates the necessity of apoA-I as cofactor of this enzyme *in vivo*, despite the fact that apoA-I is not necessary for maintaining the concentration of LCAT in plasma [35]. LCAT deficiency alone is a sufficient factor to cause HDL deficiency. Mice without apoA-I do not develop more atherosclerosis than controls when fed a regular chow diet [36]. However, there is an increase in atherosclerosis susceptibility in apoA-I-deficient mice overexpressing human apoB-100 [37]. Thus, these data support the finding in human studies that the increase in apoA-I and HDL prevents atherosclerosis; however, their deficiency does not necessarily favour atherosclerosis development unless other risk factors exist.

Finally, the study of mice genetically modified to lack apoA-I has enabled the important and specific function of apoA-I in the transport of HDL cholesterol to steroidogenic tissues to be established. In these animals, but not in mice deficient in apoA-II with a similar deficiency of HDL cholesterol, a decrease in cholesterol ester content is observed in steroidogenic tissues [34].

Among the possible actions of HDL invoked to explain their anti-atherogenic action, reverse transport of cholesterol and prevention of the oxidative modification of LDL stand out. Hence, the greater resistance of apoA-I transgenic mice to atherosclerosis may be due to an increase in these actions. In fact, it has been demonstrated that isolated HDL of apoA-I transgenic mice prevent the oxidative modification of LDL more than HDL of control mice do [38]. In contrast, the serum of apoA-I transgenic mice did not induce a greater efflux of cholesterol in Fu5AH rat hepatoma cells [39], which is the first step in reverse cholesterol transport.

4. ApoA-II

4.1. Structure and properties

The apoA-II gene is found in the q21-23 region of chromosome 1 [40]. Synthesis takes place in the liver although small quantities of RNA are also found in the small intestine [41]. The most abundant form of apoA-II in human plasma is a homodimer of 77 amino acids linked by a disulphide linkage between the cysteines in position 6 of each monomer [42]. Other less abundant molecular forms are the apoAII-apoE and apoA-II-apoD heterodimers and the monomer [43, 44]. ApoA-II has a high percentage of α-helix (62%) [45], with closer affinity than that of apoA-I for lipid monolayers. This explains why an excess of apoA-II can displace apoA-I from the HDL surface [46].

Plasma concentration of apoA-II in normolipaemic individuals is, on average, 33-35 mg/dl [47], which represents approximately 20% of HDL protein and constitutes the sec-

ond most abundant HDL apolipoprotein. Kinetic studies have demonstrated that, unlike apoA-I, the concentration of apoA-II in plasma is mainly regulated by the synthesis rate rather than by its catabolism [47].

ApoA-II function is poorly defined and its study has produced contradictory results: different studies *in vitro* have concluded that apoA-II either activates or inhibits the action of hepatic lipase (HL) [48] and CETP [49, 50]. In addition, opposing effects have been described as to whether HDL containing apoA-I and apoA-II favour or antagonise the efflux of cellular cholesterol [51, 52]. However, there is greater consensus that apoA-II is not, unlike apoA-I, a cofactor of LCAT [46, 53-56].

4.2 Mutations of the apoA-II gene

The only known case of a human apoA-II mutation is in a Japanese family with an apoA-II deficiency due to a mutation in the first nucleotide of the third intron of the apoA-II gene [57]. Despite the absence of apoA-II in two members of this family, these did not present lipoprotein changes or show signs or symptoms of atherosclerosis, suggesting that at least in humans the function/s of apoA-II can be replaced by other proteins. In fact, some animal species, such as dog, pig, cow, chicken and rabbit, have undetectable apoA-II in their plasma. In clear contrast, mice genetically modified to lack apoA-II develop HDL deficiency [58]. Thus, mouse but not human apoA-II plays a critical role in HDL.

4.3 ApoA-II transgenic animals

Overexpression of apoA-II in transgenic mice has been achieved using both the human gene and the mouse gene. The results obtained in the two cases clearly differ. Thus, it is likely that the structural differences between the two proteins have significant functional repercussions. Overexpression of human apoA-II causes the appearance of smaller HDL particles than in the plasma of control mice [56, 59]. Furthermore, mouse apoA-II and apoA-I deficiency exists in the lines of transgenic mice with greater concentration of human apoA-II (70 mg/dl). As a consequence of the apoA-I deficit, a functional deficiency in LCAT was also observed [55, 56, 60], even though LCAT concentration was normal [56, 60]. This is due to a lack of reactivity towards LCAT of apoA-II-rich HDL particles, which results in a marked decline in α-HDL particles concomitant with a mild accumulation of free cholesterol in the cornea, which resembles a condition in humans due to partial LCAT deficiency, known as Fish Eye Disease [55, 61].

It has been clearly shown that human apoA-II overexpression has a pro-atherogenic effect since it increases the atherosclerosis susceptibility of transgenic mice [61]. It also increases atherosclerosis in apoE-deficient mice [62] and, though less consistently, in double human apoA-II/simian CETP transgenic mice [63]. Moreover, joint overexpression of human apoA-II and apoA-I genes results in a partial loss of the protection to atherosclerosis conferred by apoA-I overexpression. This does not appear to be due to changes in the lipid composition of HDL, since no significant differences are observed between the HDL of apoA-I transgenic mice and of apoA-I/apoA-II transgenic mice [64].

Mice overexpressing apoA-II (mouse apoA-II transgenic mice) are characterised by increased concentrations of HDL cholesterol and larger HDL particle size than in controls [65]. As in human apoA-II transgenic mice, they show greater susceptibility to atherosclerosis, though this is more evident in a regular chow diet than in an atherogenic diet [66]. These characteristics coincide with those observed in one congenic strain selected by high expression of apoA-II [67,68].

One of the fundamental differences between human and mouse apoA-II is that the latter does not have cysteines in its structure and, thus, is found exclusively in a monomeric form. However, overexpression of human apoA-II gene mutated to not contain cysteines does not reproduce the lipoprotein phenotype of mouse apoA-II transgenic mice, suggesting that other structural differences between the two molecules are partially responsible for the different effects of their expression in mice [69].

Hence, apoA-I-rich HDL protects against atherosclerosis, but apoA-II-rich HDL does not. This could be due to various mechanisms: first, disruption of reverse cholesterol transport associated with a diminution of the efflux of cellular cholesterol and a marked impairment in LCAT activity [55, 56, 60, 61, and 70]; and second, increased susceptibility to HDL oxidation [71] and/or loss of HDL protection against LDL oxidative modification (Ribas *et al.*, unpublished observations). These alterations may have greater repercussions because of the increased plasma concentration of apoB-containing lipoproteins present in these animals due to increased VLDL production [72, 61-63, 73]. Since the overproduction of VLDL is the basic characteristic of familial combined hyperlipidaemia and that the gene of the human apoA-II is situated near (but outside) a «major» gene causing familial combined hyperlipidaemia [74], the function of apoA-II may be to block the synthesis or function of the product of the mentioned gene or the metabolic pathway in which this, as yet unknown, protein functions. This seems plausible, as various studies have demonstrated a relationship between the apoA-II plasma concentration and apoA-II locus and the apoA-II plasma concentration and the metabolism of free fatty acids and triglycerides [31, 75-77; see reference 62 for extensive discussion on this point].

5. Other HDL apolipoproteins

5.1. apoA-IV

ApoA-IV is a 46-kD glycoprotein which is mainly associated with HDL and the non-lipoprotein fraction of plasma during fasting, or with chylomicrons during the postprandial period [78]. The apoA-IV gene is mainly expressed in the intestine. There is a particularly interesting polymorphism in this gene, which determines the change of histidine to glutamine in position 360 (apoA-IV-2 allele) and is present in approximately 8% of the Western population. The most frequent form (histidine 360) is called the apoA-IV-1 allele. Individuals with an

apoA-IV-2 allele have higher concentrations of triglycerides and lower levels of HDL cholesterol in plasma than homozygotes for the apoA-IV-1 allele. In addition, they are more resistant to change in lipid concentrations in response to diets with a varied content of cholesterol and saturated fatty acids [78].

Diverse functions have been suggested for apoA-IV, some of which have already been proposed as the function of other apolipoproteins. ApoA-IV may be a regulator of the action of lipoprotein lipase on chylomicrons [79]. Patients with a hereditary deficiency of apoA-I-C-III-A-IV present vitamin E deficiency, which does not occur in patients with an inherited deficiency of apoA-I-C-III. This observation has suggested a role of apoA-IV in the absorption of liposoluble vitamins [80, 81]. Another role attributed to this apolipoprotein was as a mediator of satiety in the central nervous system. Studies carried out in apoA-IV transgenic mice found no variation in the absorption of liposoluble vitamins or in the dietary habits of these animals [82]. A possible explanation is that a limited concentration of apoA-IV is sufficient for carrying out these functions and that variations above this level have null effect. Alternatively, and given that transgenic mice overexpress apoA-IV throughout their lives, it is possible that compensating mechanisms have been developed. Analysis of knock-out mice for this gene is likely to be more informative in this respect. Lastly, apoA-IV has a stabilising effect on LCAT and activates the efflux of cholesterol in cell cultures, for which it can be an important part of the reverse cholesterol transport. In this context, it is noteworthy that apoA-IV is mainly found in particles without apoA-I and which contain LCAT and CETP [83]. However, apoA-IV not associated with lipoproteins is widely present in interstitial tissue, which is consistent with its potential action in reverse transport of cholesterol.

Human-apoA-IV transgenic mice fed a fat-rich diet present fewer lesions of atherosclerosis than control mice. Moreover, apoA-IV overexpression reduces atherosclerosis in apoE-deficient mice [84]. At least in this model, the antiatherogenic action of apoA-IV is independent of the HDL cholesterol concentration.

5.2. apoD

This apolipoprotein belongs to the superfamily of lipocalins and is found in plasma, associated mainly with HDL [44]. It is a 169-amino acid protein whose function is largely unknown. It has been postulated that it could bind ligands such as progestagens or arachidonic acid, although the physiological significance of this function and its relationship with HDL metabolism are currently unknown [85, 86].

5.3. apo E

Although this is an interesting and well-studied apolipoprotein, most of its characteristics are unrelated to a function in HDL. Of special interest for HDL metabolism is the existence of pre-β migrating HDL which contain apoE and seem able to induce the early steps of reverse transport of cholesterol from cell membranes to plasma [87]. However, since these particles are only present in individuals who have at least one apoE3 allele and not in individuals homozygous for the apoE4 or apoE2 alleles [88], apoE polymorphism could be an important determinant of reverse transport of cholesterol. In addition, the presence of apoE in HDL is related to the effectiveness of the transfer of HDL cholesterol esters to the SR-BI receptor [89].

5.4. apoJ (also called clusterine or sulphated glycoprotein 2)

This is a 427-amino acid glycoprotein of approximately 70 kD molecular weight post-transcriptionally fragmented between residues 205 and 206, which results in two units of apoJ (one containing amino acids 1-205 and the other containing amino acids 206-427). Both units remain bound through disulphide linkages [90, 91]. ApoJ is synthesised in a great variety of tissues. It is found in plasma, mainly in HDL particles which do not contain any other apolipoprotein, although at least some of these particles do also contain paraoxonase (for more information on this enzyme, see section 6.8). In some studies, a clear increase has been demonstrated in the apoJ/paraoxonase ratio in mice susceptible to atherosclerosis, and also in patients with coronary artery disease [92]. ApoJ has been demonstrated to bind to the glycoprotein 330 receptor, as do other ligands such as lipoprotein lipase or apoE [93]. Glycoprotein 330 receptor is structurally similar to the LDL-related receptor (LRP), but is mainly expressed in the liver. Given that apoJ increases in damaged tissues and exerts inhibitory activity on the C5b-C9 complex, it has been proposed that apoJ may function with glycoprotein 330 receptor to clear lipid or apoptotic residues.

5.5. apoH

This protein, also known as β2-glycoprotein I, is associated with, as well as HDL, chylomicrons, VLDL and the non-lipoprotein fraction of plasma. Although its function in lipoprotein metabolism remains unknown, it has been postulated that it has an inhibitory function in coagulation [94].

5.6. apoK-45

Little was known on this protein until its sequence was found to be identical to that of paraoxonase [95]. Its structure and function will be analysed in detail in section 6.8.

5.7. Serum amyloid-A proteins (SAA)

Serum amyloid-A proteins are a family of highly homologous molecules that meet the physico-chemical criteria for being considered apolipoproteins, and which form part of HDL. This family can be divided into two groups [96]. The first group includes amyloid proteins which increase spectacularly during the acute phase reaction that follows severe diseases or traumas. It had been suggested that these proteins displace apoA-I from the surface of HDL and can become, in the stated pathological circumstances, the main protein component of HDL. Two genes which codify for SAA1 and SAA2 exist, although there are allelic variations which are responsible for the existence of different isoforms in each

gene. The second group consists of a group of proteins (SAA4 in humans and SAA5 in mouse) which constitute 1% to 2% of HDL proteins in physiological situations.

Until recently it was believed that the functional LCAT deficiency associated with the acute phase reaction was secondary to the decreased level of apoA-I in HDL. However, overexpression of amyloid-A protein (to levels comparable to those in humans with an acute phase reaction) in apoA-I transgenic mice does not cause a reduction in HDL cholesterol or apoA-I in the absence of the mentioned reaction [97], thereby suggesting that inflammatory mediators are required to obtain this response. In contrast, overexpression of phospholipase A2 in transgenic mice, also in the absence of acute phase reaction, did increase HDL catabolism, susceptibility to oxidative modification of LDL and atherosclerosis [98-100]. Thus, the function of amyloid proteins in the metabolism and function of HDL remains to be determined.

6. Enzymes, transporters and receptor proteins involved in HDL metabolism

Different proteins that do not form part of HDL structure, such as enzymes, lipid transport proteins and receptors, are extraordinarily significant in HDL metabolism, as inherited deficiencies of these proteins clearly demonstrate. As well as the already classic lipoprotein lipase (LPL) and hepatic lipase (HL), a new member of this family of enzymes, endothelial lipase, has recently been characterised. Interesting new findings have also been reported in the field of HDL receptors.

6.1. Lipoprotein lipase (LPL)

The general characteristics of this protein are more usually analysed in relation to the metabolism of triglyceride-rich particles than that of HDL. However, different studies exist relating, albeit indirectly, LPL activity and HDL cholesterol plasma concentration [101, 102]. Also, it is known that persons with an inherited LPL deficiency (familial chylomicronaemia) have greatly reduced HDL cholesterol [103]. This is probably because LPL acts on chylomicrons and VLDL by changing their cortex/core ratio and these lipoproteins tend to recover their primitive relationship by breaking off pieces of cortex that will float in the density range of HDL2 [104]. A complementary explanation to explain the correlation between LPL activity and HDL cholesterol is that the former determines the duration of postprandial lipaemia. Further, the longer the postprandial lipaemia, the greater HDL triglycerides (through increased PTEC activity) is and, thus, increased HL activity and increased HDL catabolism (see sections 6.2 and 7).

6.2. Hepatic lipase (LH)

This enzyme is found on the surface of endothelial liver cells, from where it can be released by heparin perfusion. Its action is to hydrolyse phospholipids and triglycerides [105, 106], particularly on IDL and HDL. In the latter, it causes conversion of large (HDL2) into smaller particles (HDL3). This is due to hydrolysis of HDL triglycerides and induces dissociation of apoA-I from HDL [107]. This is likely to be a major source of very small lipid-poor HDL particles whose only apolipoprotein is apoA-I and which have electrophoretic migration of pre-β3-HDL [108]. These particles, a minor quantitative fraction of HDL –probably corresponding in part to the above-mentioned very-high-density lipoproteins (VHDL, density between 1.21 g/ml and 1.24 g/ml)–, are likely to be quantitatively the main receptors of cellular cholesterol in tests in which the rapid component of cholesterol efflux is measured.

The development of HL transgenic and knock-out animals (mice and rabbits) has confirmed and extended our knowledge of this enzyme. Rabbits and mice overexpressing HL present decreased levels of HDL cholesterol and reduced HDL particle size [109-111], while HL-deficient animals present large buoyant HDL particles enriched in phospholipids and apoE. These findings corroborate the hypothesis that HL metabolises these types of particles [112]. Double HL/apoA-II-deficient mice do not present the same degree of HDL cholesterol deficiency as apoA-II knock-out mice, which suggests that apoA-II inhibits the effects of HL on HDL metabolism in mice [113]. However, these results should be extrapolated to humans with great caution, since humans with apoA-II deficiency do not present HDL deficiency. This discrepancy could be because a good part of mouse HL binds to plasma HDL, whereas this is not the case with human HL which binds to the surface of hepatocytes. All this suggests species-specific differences in the regulation of HL.

Recent studies suggest that allelic variation in HL could account for 25% of the total variability in HDL cholesterol, at least in Western populations [114].

6.3. Endothelial lipase

This is a lipase which has only recently been cloned and analysed. It is synthesised by endothelial cells *in vitro* and is expressed *in vivo* by diverse tissues and organs, including liver, placenta, lung and kidney, but not skeletal muscle. This enzyme has a phospholipase action, mainly on HDL. Overexpression of this gene leads to a decrease in HDL cholesterol and apoA-I, thereby suggesting a functional role in HDL metabolism [115].

6.4. Lecithin: cholesterol acyltransferase (LCAT)

This is a 416-amino acid enzyme which catalyses the hydrolysis and transfer of a fatty acid from phosphatidylcholine to a cholesterol molecule (in position 3-β-OH), producing as a result cholesterol ester and lisolecithin [116]. The active site of this enzyme includes serine 181 [117-119]. This process of cholesterol ester formation occurs especially in HDL, given that apoA-I and apoA-IV are cofactors of the reaction [35, 55, 56]. The apoA-I region responsible for LCAT activation is situated in the region between amino acids 143 and 186 (repeats 6 and 7) and requires a specific conformation which is obtained during its association with lipids [120]. Part of the cholesterol esterified by LCAT stems from cell membranes,

but the main source of free cholesterol is LDL. Esterification of cholesterol is a very important step in HDL maturation and reverse cholesterol transport [121, 122].

Human LCAT has been overexpressed in three different animal models. In rabbits and monkeys, where CETP is present and active in plasma, LCAT overexpression increases plasma apoA-I and HDL cholesterol, but decreases LDL cholesterol owing to an increase in LDL catabolism *via* the LDL receptor. It also causes a reduction in atherosclerotic lesions induced by an atherogenic diet [123-125]. Overexpression of human LCAT in transgenic mice also resulted in hyperalphalipoproteinaemia [55, 126, 127], but there was no decrease in apoB-containing lipoproteins concomitant with an unexpected increase in atherosclerosis susceptibility [128]. This could be explained by the accumulation of non-functional large HDL that may be pro-atherogenic. The increased susceptibility to atherosclerosis in these mice is corrected by CETP expression [129], suggesting that the different profiles observed in several transgenic models are due to differences in CETP activity in the animals used for LCAT transgenesis.

A further important finding from the study of these genetically-modified animals is that LCAT can bind to HDL particles enriched in apoA-II. However, LCAT presents little activity towards HDL containing little or no apoA-I [35, 55-56].

In contrast, LCAT-deficient mice presented a large decrease in HDL cholesterol, apoA-I, apoA-II, and an increase in plasma triglycerides and accumulation of small nascent HDL [130-132]. Additionally, paraoxonase and platelet acetylhydrolase factor activities were lower in this model, although liver mRNA levels were comparable to those of control mice [133]. An increase in expression of the adrenal SR-BI messenger was also observed in LCAT-deficient mice [132].

Considerable experience of the consequences of LCAT deficiency has been obtained from study of patients with inherited deficiency of this enzyme. Inherited LCAT deficiencies may be either complete or partial (also known as Fish Eye Disease) [134, 135]. While plasma LCAT mass and/or activity is non-existent or very low in LCAT deficiency, there is a measurable amount of activity towards artificial and endogenous substrates in Fish Eye Disease. In both cases, however, there is a major decrease in HDL cholesterol concentration which may approach zero. However, the clinical and pathological differences between both entities are very noticeable. While the only clinical signs in partial LCAT deficiency are corneal opacities, which may seriously impair vision after the fifth or sixth decades of life, complete LCAT deficiency also causes anaemia, as the result of distortion of red cell membranes, and renal failure [135]. The mutations that cause LCAT deficiencies are distributed throughout the LCAT gene in both Fish Eye Disease and total LCAT deficiency: in both cases mutations affect both alleles [134].

Recent evidence indicates that the renal lesion in patients with total LCAT deficiency may be due to greater susceptibility to oxidative modification of phospholipids [136]. This supports the potential viability of prevention (i.e. with anti-oxidant vitamins) in patients who have been diagnosed in a pre-symptomatic period [137]. In fact, LCAT appears to help protect against oxidation of lipoproteins since it hydrolyses short polar chains of phosphatidylcholine formed during oxidation [138] and prevents lipid oxidation *in vitro* [139]. There is also evidence that lipid oxidation inhibits LCAT activity (140).

6.5. Cholesteryl ester transfer protein of cholesterol esters (CETP or LTP-1)

CETP (or Lipid-transport protein 1, LPT-1) is a 74-kD protein whose gene is located in chromosome 16q13. This protein is secreted into plasma from different tissues and is mainly linked to HDL in circulation, from where it promotes the exchange and redistribution between the different lipoprotein classes of cholesteryl esters, triglycerides and, in lesser quantity, phospholipids [141]. Given that a major part of cholesteryl esters are in HDL and most triglycerides are found in chylomicrons and VLDL, CETP activity results in a net transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins, whereas the opposite is true for triglycerides. This process, in the case of increased plasma VLDL, may be dependent on CETP, given that VLDL catabolism is faster than that of LDL and HDL. CETP action reduces the content of HDL cholesterol and increases that of apoB-containing lipoproteins. However, given that the catabolic rate of LDL and HDL is slow and similar for both lipoproteins, the result is usually independent of CETP concentration [141].

Since its identification, the role of CETP in lipoprotein metabolism and atherosclerosis susceptibility has been the subject of discussion. CETP can be anti-atherogenic by participating in cholesterol reverse transport. However, CETP action increases VLDL/LDL cholesterol and HDL triglyceride (an HL substrate) and could therefore be pro-atherogenic [141].

Studies performed on CETP transgenic mice have not clarified matters. Simian CETP expression in control mice and in human apoA-II transgenic mice [63, 142] and that of human CETP in apoE-deficient mice [143] increased atherosclerosis susceptibility. In contrast, CETP expression in other hypertriglyceridaemic mice, like those overexpressors of apoC-III, induced a reduction in atherosclerosis susceptibility [144]. Also, CETP expression prevented atherosclerosis in human LCAT transgenic mice [128]. One interpretation of these results could be that CETP prevents atherosclerosis when it improves reverse cholesterol transport (i.e. in the case of double LCAT/CETP transgenic mice).

However, at least two studies have demonstrated the anti-atherogenic potential of inhibiting CETP in rabbit, an animal with high activity of this protein in its plasma [145, 146].

Inherited CETP deficiencies have been described in humans and appear to be relatively frequent in the Japanese population (up to 5% of the population is heterozygote for this deficiency) [147]. In the Honolulu Heart Study, an excess of coronary disease was confirmed in subjects with CETP deficiency. When CETP deficiency was associated with an HDL-cholesterol concentration between 1.06 and 1.55 mmol/L, coronary risk was higher than in subjects with similar HDL cholesterol levels but without CETP deficiency [148]. However, there was no increase in coronary risk for

those deficient in CETP when HDL cholesterol was above 1.55 mmol/L [147]. In this event, the benefits of high HDL cholesterol overcome the effects of impaired reverse cholesterol transport due to CETP deficiency.

It has recently been demonstrated that CETP synthesis is induced by the interaction of the LXR (liver X receptor) with the CETP gene promoter [149]. The fact that this transcription factor also regulates the promoter of 7α-hydroxylase cholesterol, the key enzyme in the synthesis of biliary acids from the cholesterol delivered to the liver by HDL, could be considered evidence of the molecular coordination of the different steps of reverse cholesterol transport [148].

6.6. Phospholipid transporter protein (PLTP and/or LTP-2)

This is a protein of 476 amino acids with a molecular weight of 54.7 kD, to which N-glycosylation gives an apparent molecular weight of 75 kD. Prior to its characterisation, it was referred to as a converter activity because of its ability to induce changes in HDL size [150]. PLTP secretion depends on the segment comprised between residues 35 and 50, and also on intracellular folding of the protein which is induced by a disulphide linkage between cysteines 129 and 168 [151, 152]. Its association with HDL is due to the binding of PLTP to apoA-I, near residues 27 to 48 of this apolipoprotein [153]. PLTP is synthesised in ovary, thymus and placenta and to a lesser degree in the majority of other organs. Its function consists of transferring phospholipids from some HDL particles to others, thereby causing displacement of apoA-I from the HDL surface and, thus, generation of smaller HDL particles (pre-β1 HDL) [154, 155]. The resulting HDL particles tend to undergo fusion, which explains why PTLP also generates larger HDL particles [56, 157].

As studies performed in cholesterol-loaded fibroblasts show, PLTP may play an important role in cellular cholesterol and phospoholipid efflux by increasing binding of HDL to cells. This efflux is HDL-specific and is not seen if only albumin or purified apoA-I are found in the medium, or in the case of fibroblasts of patients with Tangier disease [158]. PLTP can additionally transfer free cholesterol, α-tocopherol and lipopolysaccharide, which suggests that it may also be involved in anti-microbial defence [159]. Its capacity to transfer α-tocopherol from HDL and complexes of α-tocopherol-albumin towards LDL and endothelium may indicate a function in the prevention of LDL oxidative modification and an additional entry pathway of α-tocopherol to endothelial cells [160].

Another interesting property of this protein is its proteolytic serine-esterase activity, which is able to cut the carboxy terminal extreme of apoA-I (28.2 kD), leaving a fragment of 22.9kD. This proteolytic activity acts on both free apoA-I and apoA-I/phospholipid complex of density greater than 1.250 g/ml, showing an induction phase (or lag phase) of 8-10 hours at 37ºC after which proteolysis occurs in a time-dependent manner [161]. The physiological significance of this activity is unknown, but it has been suggested that it may affect HDL metabolism by generating an apoA-I compartment with increased catabolism.

The generation of PLTP-deficient mice has provided *in vivo* evidence of the importance of this protein in HDL metabolism. These mice show a decrease in plasma phospholipids, cholesterol and apoA-I HDL when they are fed a regular chow diet. However, when these animals are fed a fat-rich diet, they present increased plasma phospholipids, free and esterified cholesterol associated with VLDL and LDL without changes in apoB content [162]. In another study, these animals showed accelerated HDL catabolism as well as accumulation of lamellar apoA-IV-rich lipoproteins [163]. These results support a role of PLTP activity in the transfer of phospholipids and free cholesterol from triglyceride-rich lipoproteins to form nascent HDL.

Human PLTP transgenic mice only showed phenotypic effects after breeding with human apoA-I transgenic mice. In this case, increased phospholipids and HDL cholesterol were found in plasma due to an increase in pre-β HDL [164]. In another study in which PLTP was overexpressed in mice (between 2.5 and 4.5 times more than in controls), their plasma showed a 2-3-fold increase in pre-β HDL and was more efficient than control plasma in preventing cholesterol accumulation in macrophages [165].

PLTP overexpression by adenovirus in control or apoA-I transgenic mice caused, nevertheless, a marked decrease in HDL cholesterol and the appearance of some HDL of greater size than in control mice [166]. Studies of alcoholic patients, before and after giving up alcohol, showed that while CETP activity tends to increase small HDL particle formation, the predominant action of PLTP is the opposite [167].

6.7. Cholesterol efflux regulatory protein (CERP)

Several groups [168-171] have recently cloned and sequenced this protein. Based on theoretical predictions, it is a protein with 12 transmembrane domains and two intracellular ATP binding sites, which could provide energy for the transfer of ligands. In this model, the protein presents two halves, with six transmembrane helices, each bound by a large hydrophobic segment containing a regulatory region [172] (Figure 2).

This protein was identified and characterised during the study of the defective gene in Tangier disease. Those affected by this rare inherited disease have extremely low HDL levels and show an accumulation of cholesterol esters in endothelial reticulum cells, which is the origin of some of the clinical characteristics of the disease, such as hepatomegaly, spleen enlargement, orange-coloured tonsils [173] and a higher risk of atherosclerosis. Tangier disease is caused by defects in the ATP-binding cassette transporter-1 gene (ABC-1 and/or ABCA-1, according to the Human Gene Nomenclature Committee) which codifies for CERP. Furthermore, mutations of this gene have been found in patients with familial HDL deficiency, suggesting that Tangier disease and familial HDL deficiency are allelic manifestations of mutations of the same gene [168]. Marked impairment of CERP function (i.e. mutations in homozygosis or compound heterozygosis) may lead to the most severe form (accumula-

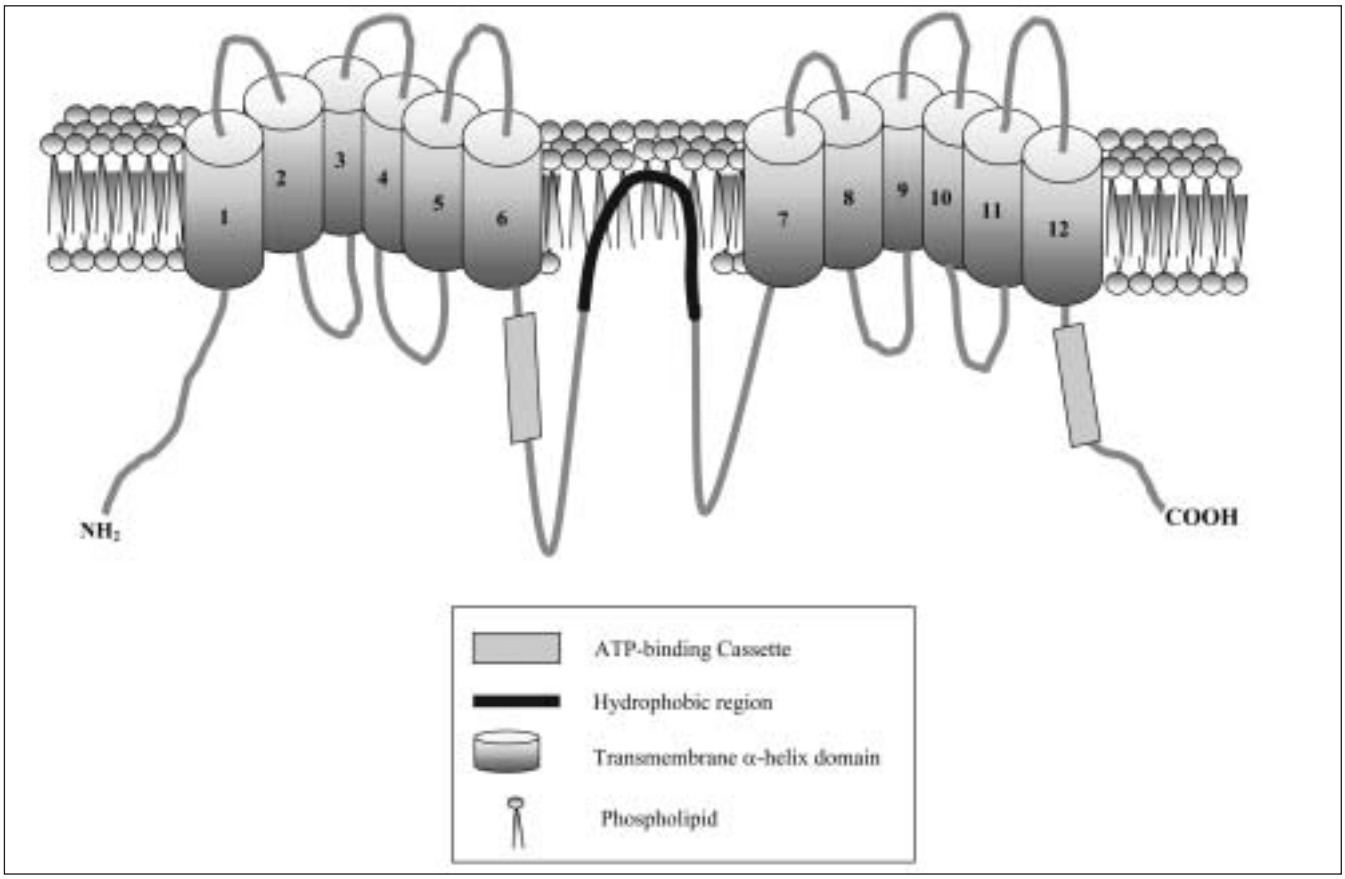

Figure 2. A model of the product of the ATP-binding cassette protein-1 (ABC-1), known as the cholesterol efflux regulatory protein (CERP), which is defective in Tangier disease and familial HDL deficiency.

tion of cholesterol in tissues and HDL deficiency which characterise Tangier disease), while less impairment (i.e. in heterozygotes) may cause HDL deficiency but without the clinical manifestations of massive cholesterol accumulation in tissues (familial HDL deficiency).

CERP defects cause a decreased efflux of cellular phospholipids and cholesterol towards HDL, thus severely impairing reverse lipid transport and HDL formation. CERP-deficient mice show a severe HDL deficiency and other alterations consistent with the phenotype of patients affected by Tangier disease. In both, the transport of lipids from the Golgi to the plasma membrane is defective [174, 175].

CERP is found in the plasma membrane, which is consistent with its possible function as transporter of lipids to HDL [176]. CERP expression is regulated. It is absent in proliferating cells, but is induced by quiescence, especially in cholesterol-loaded macrophages [176]. CERP presents a consensus region of kinase proteins dependent on cAMP, suggesting that phosphorylation of the protein may have functional effects. Consistent with this, cAMP has been described as increasing both CERP mRNA and protein expression on the cell surface [176]. Furthermore, cAMP analogues induce apolipoprotein-mediated lipid efflux. It has been demonstrated that apoA-I binds to a membrane protein of molecular weight identical to CERP [177] and that IL-1β stimulates the apoA-I-dependent efflux of cholesterol and phospholipids, which suggests that at least some cytokines can modulate CERP [178].

CERP probably transfers free cholesterol and phospholipids between the internal and the external parts of the plasma membrane, where they are captured by apoA-I for HDL formation. Although it is not yet known whether these are their true ligands or whether other cell proteins are involved [179], their importance in cell lipid efflux towards HDL is evident.

Other results suggest that CERP participates in the phagocytosis of apoptotic cells and secretion of IL 1-β by macrophages [180].

6.8. Paraoxonase

This enzyme belongs to the group of type A esterases. It is able to hydrolyse diverse organophosphates, which explains the interest of toxicologists in it. Paraoxonase is a protein of approximately 45 kD, whose gene is situated in the long arm of chromosome 7. The paraoxonase gene (also called PON1) is part of a family which has at least three related genes that share a high degree of homology and which are grouped together (the others are called PON2 and PON3) in the same chromosome region, thereby suggesting gene duplication [181]. Paraoxonase is mainly synthesized and secreted by the liver and is associated with HDL in plasma, especially in particles containing apoA-I and apoJ [182]. A special characteristic of this protein is the retention of the hydrophobic signal in the N-terminal end, which is responsible for its union with HDL through phospholipid bind-

ing [183]. Given that it is unlikely that the function of paraoxonase is to metabolise synthetic substances, the question is: what is its function? Paraoxonase protects against inflammation in cells of the arterial wall, probably by destroying biologically active lipids from oxidised lipoproteins. A possible mechanism could be the hydrolysis of lipid peroxides in oxidised lipoproteins, especially hydroperoxide phospholipids [184]. On the other hand, a calcium-dependent HDL enzyme, homocysteine:thiolactone hydrolase –which is known to protect against one of the potential toxic effects of hyperhomocysteinaemia, the accumulation of homocysteine thiolactone– has recently been purified and found to be identical to paraoxonase [185].

Various polymorphisms of the paraoxonase gene have been described. One is associated with a different activity of the enzyme using paraoxon as a substrate. While isoform A (low activity) has Gln in position 192, isoform B (high activity) has Arg in the same position [186, 187]. However, some studies found an increase in genotypes AB and BB in patients with coronary artery disease [188, 189], though others found no differences in the genotype distribution of patients and controls [190, 191]. The explanation could be the greater protective effect of paraoxonase A (low activity against paraoxon) than of B (high activity against paraoxon) on lipoprotein oxidation [192]. Thus, the active centre responsible for paraoxonase/arylesterase activity is not the same as the centre responsible for protection against oxidation. Protective activity against oxidation seems to involve the free cysteine of paraoxonase (Cys 284) and does not require the N terminal domain, which is responsible for HDL binding [192].

Another polymorphism affects amino acid 55 of paraoxonase which can be a methionine or a leucine. In one study, homozygosity for leucine in position 55 was found to be an independent risk factor for cardiovascular disease [193], though this has not been confirmed in other studies [194].

Polymorphisms which influence the plasmatic concentration of the enzyme have also been described in the promoter of the paraoxonase gene. These polymorphisms are situated in positions -907, -824 and -107, the last of which may be important for gene expression [195]. By affecting the plasma concentration of paraoxonase, these polymorphisms could contribute to the study of the protective capacity of paraoxonase against atherothrombotic cardiovascular disease.

The development of paraoxonase-deficient mice has revealed a clear relationship between paraoxonase expression and the formation of atherosclerotic lesions. Besides being more susceptible to organophosphates, as expected, paraoxonase-deficient mice were more susceptible to atherosclerosis than control mice. Both LDL and HDL isolated from these mice were more susceptible to oxidation than LDL and HDL of control mice [196]. These findings, increased susceptibility to oxidation and to atherosclerosis, have been replicated in double apoE/paraoxonase-deficient mice [197].

Non-genetic factors may affect paraoxonase concentration and activity. In the strain of mice susceptible to atherosclerosis C57BL/6J, a decrease in paraoxonase in plasma was observed after eight weeks of atherogenic diet. This effect was not observed in the strain of mice genetically resistant to atherosclerosis C3H/Hej [198]. In apoE-deficient mice, ageing increases lipid peroxidation, which is concomitant with decreased paraoxonase activity [199], while the addition of antioxidants to their diet preserves paraoxonase activity [200]. A decrease in paraoxonase activity in the plasma of human volunteers is also observed four hours after a meal rich in fried oils [201]. These observations suggest that dietary and environmental factors also modulate the activity of paraoxonase in plasma.

During the acute phase response, a change in HDL protein composition occurs, which is associated with decreased paraoxonase and platelet activating factor acetylhydrolase activities [202]. Acute phase response induced by lipopopolysaccharide injection in hamsters causes a decrease in both plasma activity and paraoxonase mRNA. This is mediated by the pro-inflammatory cytokines TNFα and IL-1 [203]. It is thus possible that acute repetitive or chronic inflammation could be pro-atherogenic.

Finally, it is relevant that paraoxonase 3 (PON3) has been cloned and expressed, for this shows that, in rabbits at least, PON3 protects against oxidation more powerfully than paraoxonase 1 [204].

6.9. Platelet activating factor acetylhydrolase (PAF-AH)

This calcium-independent serine esterase is associated with LDL (especially the dense LDL of 1.050<d<1.063 g/ml) and with HDL, 70% and 30%, respectively. The nature of PAF-AH binding to LDL has been studied by directed mutagenesis, which has shown the importance of residues 205, 115 and 116 of PAF-AH and the C-terminal extreme of apoB-100 of LDL in this binding. This occurs in human LDL, while in mice there is no association of PAF-AH with LDL and the greater part of this enzyme is associated with HDL [205].

PAF-AH acts by hydrolysing the sn-2 ester bond of the platelet activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) and liberating acetate. This enzyme also hydrolyses the minimally-oxidised peroxide fatty acids of LDL phospholipids, action which prevents endothelial cells from segregating inflammatory factors promoting monocyte binding [206]. Oxidised phospholipids could be transferred from LDL to HDL, where they could be inactivated by hydrolysis. PAF-AH action in HDL prevents the peroxidation of LDL; and this is not prevented if a serine esterase inhibitor is used [207]. Apparently, PAF-AH and paraoxonase act jointly in lipoprotein oxidation protection, as suggested by the fact that PAF-AH is not effective in preventing LDL oxidation in paraoxonase-deficient mice [208].

LDL oxidation causes PAF-AH activity to decrease, probably due to the formation of a non-competitive inhibitor [209].

6.10. HDL receptors

Though various authors have identified proteins that bind HDL in membranes of fibroblasts, hepatocytes, adipocytes

and macrophages, the physiological function of many of these is still unknown. Therefore, in this review we will focus on two receptors, one of which (cubilin) is able to internalise HDL particles as well as apoA-I, whereas the other (named CLA-1 or SR-B1, depending on the authors) captures HDL cholesteryl esters without internalisation of the lipoprotein particle.

Cubilin is expressed in the intestinal epithelium, kidney and placenta, and mediates catabolism of small HDL and lipid-poor apoA-I. Previously, cubilin had been described as an intestinal receptor of the complex vitamin B12-intrinsic factor [210, 211]. Thus, it is likely to be a «multi-ligand» receptor. Cubilin is a protein of 400 kD. As it does not contain any transmembranous segment in its structure, it needs the co-expression of another protein, megalin, which is a member of the LDL receptor family and does contain a transmembrane segment. This suggests that it acts as a co-receptor with cubilin. Both cubilin and megalin are expressed in the proximal renal tubule. In the kidney, their function could be the reabsorption and degradation of small HDL and lipid-poor apoA-I from the glomerular filtrate. In the intestine, cubilin is important for the absorption of vitamin B12, and in the placenta it could be the transport pathway of cholesterol from the mother to the foetus [212].

SR-BI (scavenger receptor BI) [213] or CLA-1 [214] is a protein of 57 kD whose theoretical structure contains two cytoplasmatic domains, a large extracellular region and two transmembrane domains [215]. SR-BI is expressed in liver, ovaries and adrenal glands [214], which led researchers to hypothesise on its possible function, especially as these tissues are known to capture HDL cholesteryl esters. The receptor seems to be located in caveolae, at least in some cell types [216], while in other studies an association was found in other domains of the plasma membrane where caveolae had not been identified [217]. An isoform of this receptor, named SR-BII, has a different C-terminal domain due to alternative splicing. This isoform is less efficient in capturing HDL cholesteryl esters, although its function is largely unknown [218].

The selective transfer of HDL cholesteryl esters to cells appears to need direct binding of the HDL particle to SR-BI, probably by recognition of amphipathic helices characteristic of soluble apolipoprotein [219] such as apoA-I, apoA-II, apoC, although SR-BI can also bind anionic phospholipids of vesicles that do not contain proteins [220]. Once an HDL particle is bound to the SR-BI surface, it has been postulated that a hydrophobic canal would be formed and cholesteryl esters would cross the cell plasma membrane following a favourable gradient [221]. The role of SR-BI in this transfer seems to be more important than the simple binding of HDL near the plasma membrane, since a related receptor, CD36, also binds HDL but is incapable of mediating such a net transfer of cholesteryl esters. It has been suggested that the extracellular domain of SR-BI is responsible for generating such a hydrophobic canal [222].

SR-BI also promotes cell efflux of free cholesterol. It has been suggested that this phenomenon is not only due to HDL binding but is also dependent on the change of membrane lipid domains which facilitate a bi-directional flow of free cholesterol [223].

SR-BI activity in the selective recruitment of cholesteryl esters may be important in the liver during the final steps of cholesterol reverse transport. Mice overexpressing liver SR-BI present very rapid clearance of HDL particles as well as an increase in biliary cholesterol [224]. Furthermore, a mutation in the SR-BI promoter of mice, which reduces the expression of this receptor by half, results in increased plasma concentration of HDL cholesteryl esters as well as increased HDL particle size [225]. These effects are more dramatic in mice completely deficient in SR-BI. Moreover, these mice present a decrease in adrenal cholesterol [226] and female infertility. This suggests that the SR-BI-mediated pathway is important for the production of steroid hormones by adrenal glands.

Other murine models presenting an HDL deficiency, such as those of mice deficient in LCAT [132], apoA-I and HL [227], also present a concomitant increase in SR-BI receptor expression in adrenal glands, apparently of a compensatory nature since the increase is parallel to a drop in cholesteryl esters in this tissue.

Oestrogens and dietary cholesterol regulate SR-BI expression in liver, while regulation of the expression in adrenal glands is mediated by the hypothalamic-pituitary-adrenal axis, mainly through ACTH [228].

The relationship between this receptor and atherosclerosis has also been studied by inducing genetic modifications in mice. Double apoE/SR-BI-deficient mice present much more rapid atherosclerosis than apoE-deficient mice [229] and, consistently, LDL receptor-deficient mice overexpressing SR-BI present fewer atherosclerotic lesions than LDL receptor-deficient mice [230]. It has been suggested that part of the SRB-I protection against atherosclerosis could be due to its ability to bind apoB-containing lipoproteins, in addition to its action on reverse cholesterol transport. It is noteworthy that SR-BI mediates the selective transfer of HDL-oxidised cholesteryl esters to the liver and may be responsible for their rapid clearance, which could contribute to their antiatherogenic effect [231].

There are several polymorphisms of the human SR-BI locus which are associated with variations in lipid and anthropometric parameters [232], which suggests that this receptor helps regulate body weight.

Recently, the increased plasma HDL-cholesterol concentration present in Ob/Ob mice (deficient in leptin) has been shown to be due to a drop in HDL catabolism (binding, association, degradation and re-secretion) in hepatocytes. This implies decreased recruitment of HDL cholesteryl esters, even though SR-BI expression is not altered [233]. The molecular mechanisms involved are unclear; thus, whether there are one or more HDL receptors other than SR-BI and megalin/cubilin, and whether leptin regulates the function of one or more HDL receptors, remain to be clarified.

7. Metabolism of HDL: determinants of HDL cholesterol plasma concentration

HDL synthesis occurs at different levels: hepatic, intestinal and circulating plasma. ApoA-I and apoA-II are synthesised in the liver and seem to be secreted as lipid-poor particles or even as free apolipoproteins. These apolipoproteins capture phospholipids and cholesterol from cells or bind lipids originated by lipolysis-induced remodelling of triglyceride-rich lipoproteins. These discoidal lipoprotein particles are later converted into mature spherical HDL by LCAT activity [83, 234]. The intestine synthesises and secretes HDL particles with apoA-I with or without apoA-IV, but without apoA-II (the principal synthesis site of this apolipoprotein is the liver). Finally, a part of HDL is synthesised during the catabolism of triglyceride-rich lipoproteins.

Various clinical studies have demonstrated that post-heparin LPL activity correlates with the concentration of HDL2 cholesterol [102]. However, the mechanism by which this is produced is unknown, since the surface material liberated from triglyceride-rich lipoproteins is probably denser than HDL2. One possibility is that this surface material binds apoA-I and phospholipids and matures by the action of LCAT reaching the density characteristic of HDL2. Another possibility is that this surface material undergoes fusion with pre-existing HDL particles.

Now that the structure and composition of HDL, as well as the main related enzymes, lipid transport proteins and receptors involved in their metabolism, have been reviewed, it is now possible to review the most contrasted aspects of HDL metabolism, with special emphasis on the processes that modulate the interconversion of different subtypes of HDL (Figure 3).

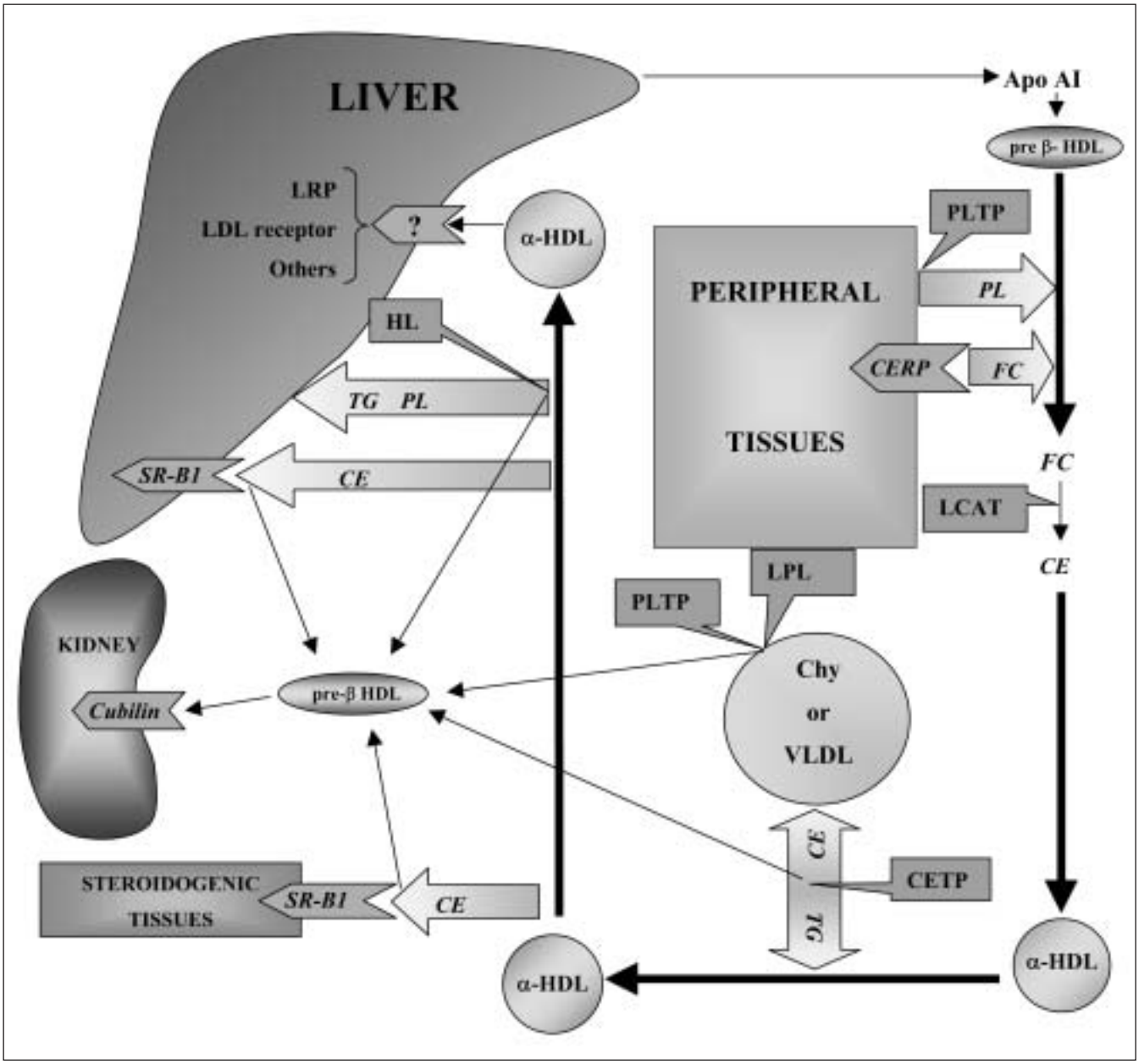

Figure 3. Summary of HDL metabolism with particular reference to reverse cholesterol transport.

HDL catabolism is the main determinant of HDL plasma concentration in the population. The main determinant of HDL catabolism appears to be lipid composition and particle size, which are highly dependent on their interaction with lipolytic enzymes and transfer lipid proteins [235]. Both these parameters, lipid composition and particle size, are closely related. In most pathophysiogical circumstances, a very close correlation exists between plasma concentrations of apoA-I and HDL cholesterol. Despite this, it is clear that the destination of part of the lipid and protein components of a given HDL particle differs from that of the lipoprotein particle [236, 237]. This is due to a rapid substitution of some lipid and protein components of HDL. This occurs through, first, the phospholipase and the triglyceride-lipase actions of HL; second, the action of lipid transfer proteins (LTP-1 or CETP and LTP-2), which transfer cholesteryl esters, triglycerides and phospholipids (in the case of CETP) or only phospholipids (in the case of LTP-2) to other lipoprotein particles; and lastly, the specific transfer of cholesteryl esters to cells in a process mediated by specific receptors (SR-BI) which recognise HDL and lipid-poor apoA-I in liver, adrenal gland and ovaries.

The actions of these enzymes and lipid transport proteins on HDL induce the dissociation of part of the apoA-I molecules contained in a given HDL particle. Lipid-poor or lipid-free apoA-I may form HDL particles by inducing reverse cholesterol transport or, alternatively, be filtered in the kidney and catabolised at tubular level by the megalin/cubilin complex [211, 212, 237]. The renal catabolism of free apoA-I explains why its clearance from plasma is higher than for the whole HDL particle.

Factors which tend to increase or decrease HDL concentration are shown in Table 3. As previously mentioned, the most frequent mechanism involved in decreased plasma HDL cholesterol is increased HDL catabolism. This is the case in hypertriglyceridaemia, where the increase in HDL catabolism seems to result from the increase in either the number of triglyceride-rich particles, its lipid content, or both. Both situations will produce HDL triglyceride enrichment by CETP. This will increase HL action on HDL, decrease HDL particle size and increase apoA-I dissociation from the HDL surface [102, 238]. There is an inverse relationship between HDL particle size and their clearance [235], with the small HDL particles being more rapidly catabolised.

Table 3. Factors influencing the plasma concentration of HDL cholesterol

Raises HDL cholesterol	*Lowers HDL cholesterol*
Sex: female	Sex: male
Exercise	Sedentary habits
Alcohol	Abdominal obesity
Saturated fat-rich diets	Saturated low-fat diets
CETP deficiency	Polyunsaturated fat-rich diets
Hepatic lipase deficiency	Hypertriglyceridaemia
Oestrogens	Progestagens, androgens
Niacin	Probucol
Fibrates	Smoking
Phenytoin	

One of the genetic determinants of HDL cholesterol concentration in the general population has been situated in the gene whose defect causes Gaucher's disease. Mutations of this gene in heterozygosis could cause 19.5% of the genetic variability of HDL cholesterol, at least in the Spanish population [239]. Whether this gene affects HDL synthesis or catabolism is, at present, unknown.

The same mechanisms that explain decreased HDL cholesterol in individuals with hypertriglyceridaemia, i.e. decreased LPL activity and/or increased HL and CETP activities, may explain decreased HDL cholesterol in individuals without hypertriglyceridaemia. In some cases, changes in the concentration and/or activity of these proteins have an important genetic basis. Decrease in HDL cholesterol due to a decrease in the LPL/HL ratio appears to be more strongly associated with coronary disease than CETP increases [240].

It is noteworthy that not all causes of increased HDL cholesterol shown in Table 3 can be associated with greater protection against atherosclerosis, and the reverse is also true for the causes of decreased HDL cholesterol. For example, the increase in saturated fat intake increases HDL cholesterol, which occurs in a pro-atherogenic context. In addition, some cases of increased HDL cholesterol (hyperalphalipoproteinaemia) due to inherited HL or CETP deficiencies could be associated with a greater risk of atherosclerosis, although this is in part in the former case due to the increased concentration of remnant lipoproteins [241, 242]. Some inherited HDL deficiencies do not appear to be associated with greater atherogenic risk. Thus, although a clear epidemiological relationship exists between levels of HDL cholesterol and atherogenic risk, exceptions to this rule do exist.

8. HDL functions of potential anti-atherogenicity

The effect of expressing or deleting several genes whose effects are related to HDL metabolism and atherosclerosis susceptibility is shown in Table 4.

Numerous functions which could have physiological significance have been ascribed to HDL. Some of these are listed in Table 5. However, at present there are no data indicating the relative contribution of these functions to the total anti-atherogenic capacity of HDL. Two of the most anti-atherogenic functions are examined in detail below.

8.1 Reverse cholesterol transport

This term indicates the process by which cholesterol originating from peripheral cells is captured by HDL to be delivered to the liver, from where it will be partly eliminated through the biliar pathway. This hepatic clearance of cholesterol is, in humans, carried out mainly through VLDL, IDL and LDL. The process by which cholesteryl esters are transported from HDL to apoB-containing lipoproteins (chylomicrons, VLDL, IDL and LDL) is mediated by CETP. Three processes

Table 4. Modification of HDL-related genes in experimental animals: effects on HDL cholesterol and atherosclerosis susceptibility.

Animal Species	*Modification*	*HDLc level*	*Atherosclerosis*	*Reference*
Mouse	Apo AI KO	Decreased	Unchanged	**36**
Mouse	ApoA-I KO/H apoB transgenic	Increased	Decreased	**37**
Mouse	H ApoA-I transgenic	Increased	Decreased	**7**
Mouse	Double H ApoA-I/Apo(a) transgenic	Unchanged	Decreased	**8**
Mouse	H ApoA-I transgenic/apoE KO	Increased	Decreased	**10**
Rabbit	H apo AI transgenic	Increased	Decreased	**32**
Mouse	Apo AII KO	Decreased	ND	**58**
Mouse	Apo AII transgenic	Increased	Increased	**66**
Mouse	H apo AII transgenic	Decreased	Increased	**60**
Mouse	H ApoA-II transgenic/apoE KO	Decreased	Increased	**62**
Mouse	Double H ApoA-II/ simian CETP transgenic	Decreased	Increased	**63**
Mouse	H Apo AIV transgenic	Unchanged	Decreased	**84**
Mouse	H hepatic lipase transgenic	Decreased	Decreased	**109**
Mouse	Hepatic Lipase KO	Increased	ND	**112**
Mouse	Hepatic Lipase/Apo AII-double KO	Decreased	ND	**113**
Mouse	H Endothelial Lipase adenovirus expression	Decreased	ND	**115**
Rabbit	H-LCAT transgenic	Increased	Decreased	**123**
Mouse	H LCAT transgenic	Increased	Increased	**128**
Mouse	Double H LCAT/ CETP transgenic	Increased	Decreased	**129**
Mouse	LCAT KO	Decreased	ND	**132**
Mouse	Simian CETP transgenic	Decreased	Increased	**142**
Mouse	Double H CETP /Apo AI transgenic	Decreased	Increased	**144**
Mouse	Triple H CETP / Apo AI / Apo CIII transgenic	Decreased	Decreased	**144**
Mouse	PLTP KO	Decreased	ND	**163**
Mouse	H PLTP transgenic	Increased	Decreased	**165**
Mouse	Double H PLTP/ Apo AI transgenic	Increased	ND	**164**
Mouse	ABCA-1 KO	Decreased	Increased	**175**
Mouse	paraoxonase KO and double paraoxonase/apoE KO	Unchanged	Increased	**196, 197**
Mouse	SR-BI KO	Increased	ND	**226**
Mouse	Double SR-BI / apoE KO	Increased	Increased	**229**

Abbreviation not used in the text: H, human

are involved in the reverse cholesterol transport: cholesterol efflux from cells to plasma, cholesterol esterification and destination of preformed cholesteryl esters (see Figure 3).

8.1.1. Cholesterol efflux

Two theories exist on cellular cholesterol efflux to HDL. The first is that this efflux is a diffusive, slow, receptor-independent process. HDL particles which capture cholesterol do not have any special characteristic (HDL with apoA-I and without apoA-II and HDL with apoA-I and apoA-II are equally capable of inducing this cholesterol efflux) [155, 243] (Figure 3). A second theory proposes that cholesterol efflux is receptor-dependent and that HDL with apoA-I and without apoA-II are more efficient than HDL with apoA-I and apoA-II [155, 243]. Some authors have even suggested that HDL particles with apoA-I and apoA-II not only are not capable of promoting cholesterol efflux, but also block the process by competing for binding to the receptor [244, 245].

The current belief is that both processes can co-exist, and that in some cell types, cholesterol efflux occurs using both systems, while in others one of the efflux systems is predominant. However, there has been progress in defining a mechanism of rapid and specific cholesterol transport to a minor fraction of HDL, the pre-β HDL. This type of particle only contains apoA-I, and accounts for only 5% of total HDL. Its ability to recruit cholesterol is lost, to a great extent, if cultured cells are previously treated with proteases, indicating specificity though not demonstrating a specific interaction mediated by receptors [243]. It is supposed that the type of interaction between the pre-β HDL and a hypothetical receptor induces cholesterol translocation from the cytoplasm to the cell membrane and could correspond to CERP and, perhaps, to other members of the ABC protein group [176, 179].

Three types of pre-β HDL exist. The pre-β HDL1s are the smallest particles and those which first receive cell cholesterol. After a few minutes, the cholesterol passes to pre-β HDL2 and to pre-β HDL3, which are particles increasingly

Table 5 . Potential anti-atherogenic HDL functions.

Reverse cholesterol transport
Prevention of LDL oxidative modification
Inhibition of LDL aggregation (*)
Suppression of the inhibition of the endothelial relaxing factor (*)
Prostacycline production and stability (*)
Inhibition of macrophage migration (*)
Inhibition of the expression of adhesion molecules by endothelial cells (*)
Increase in protein S and C activities (*)

* See references 264-270 for details

greater in size. Authors differ as to whether the major part of cholesterol is mainly esterified in pre-β HDL and transferred to α-HDL and LDL, or whether a greater part is transferred unesterified to α-HDL first and later to LDL [54, 243, 246]. According to this latter theory, free cholesterol is re-transferred to α-HDL (mainly HDL3), where it is esterified by LCAT. In short, this esterification process is a key step for maintaining the gradient of transfer from LDL to HDL. In support of the latter hypothesis, the principal source of non-esterified cholesterol for HDL is LDL and not cells [243].

8.1.2. Cholesterol esterification

Plasma cholesterol esterification is catalysed by LCAT. The importance of LCAT action for the two cholesterol efflux systems postulated varies. Complete LCAT deficiency does not have the same impact on all tissues. Furthermore, there is experimental evidence indicating that LCAT activity is not necessary to produce the rapid and specific component of cholesterol efflux, which is directed, in the first place, to HDL particles of pre-β mobility. However, LCAT activity is necessary for the slowest non-specific component of cholesterol efflux, which is directed to HDL particles in a concentration-dependent fashion [247]. In this regard, it has been hypothesised that the reverse of LCAT deficiency (i.e. a disease which principally affects the cell lines which depend fundamentally on reverse rapid and specific transport of cholesterol) is Tangier disease [247, 141]. This disease is characterised by accumulations of cholesteryl esters, especially in cells derived from the reticulum-endothelial system [142].

8.1.3 Destination of cholesteryl esters formed in HDL

Destination of cholesteryl esters formed in HDL is varied. These esters can be: i) captured by tissues together with the whole HDL particle, especially in the liver and kidney; ii) captured in a specific way from HDL after their binding to the SR-BI receptor (this process does not imply capture of the HDL particle by the cell) in the liver, gonads and adrenal glands; and iii) transferred by CETP to apoB-containing lipoproteins which are later captured by the cells *via* a receptor-mediated mechanism (LDL receptor, VLDL receptor, LRP, gg330, etc).

HDL metabolism, with special reference to the reverse cholesterol transport process, is summarised in Figure 3.

8.2. Prevention of oxidative modification of LDL

Oxidised modification of HDL inhibits reverse cholesterol transport and blocks LCAT activity [248-250]. Thus, oxidative modification of HDL could inhibit one of the anti-atherogenic functions of HDL such as reverse cholesterol transport. However, current research focuses on the ability of HDL to inhibit LDL oxidative modification, a phenomenon that appears to be vital in the origin of atherosclerosis [251, 252]. The mechanism of this inhibition seems to be, at least in part, enzymatic. By means of poorly defined interactions between LDL and HDL, HDL enzymes –such as the coaction of paraoxonase and PAF-AH– convert oxidised fatty acids with pro-atherogenic properties into others which have no biological activity [184, 207] (see Figure 4 for a view of this hypothetical anti-atherogenic mechanism). This function

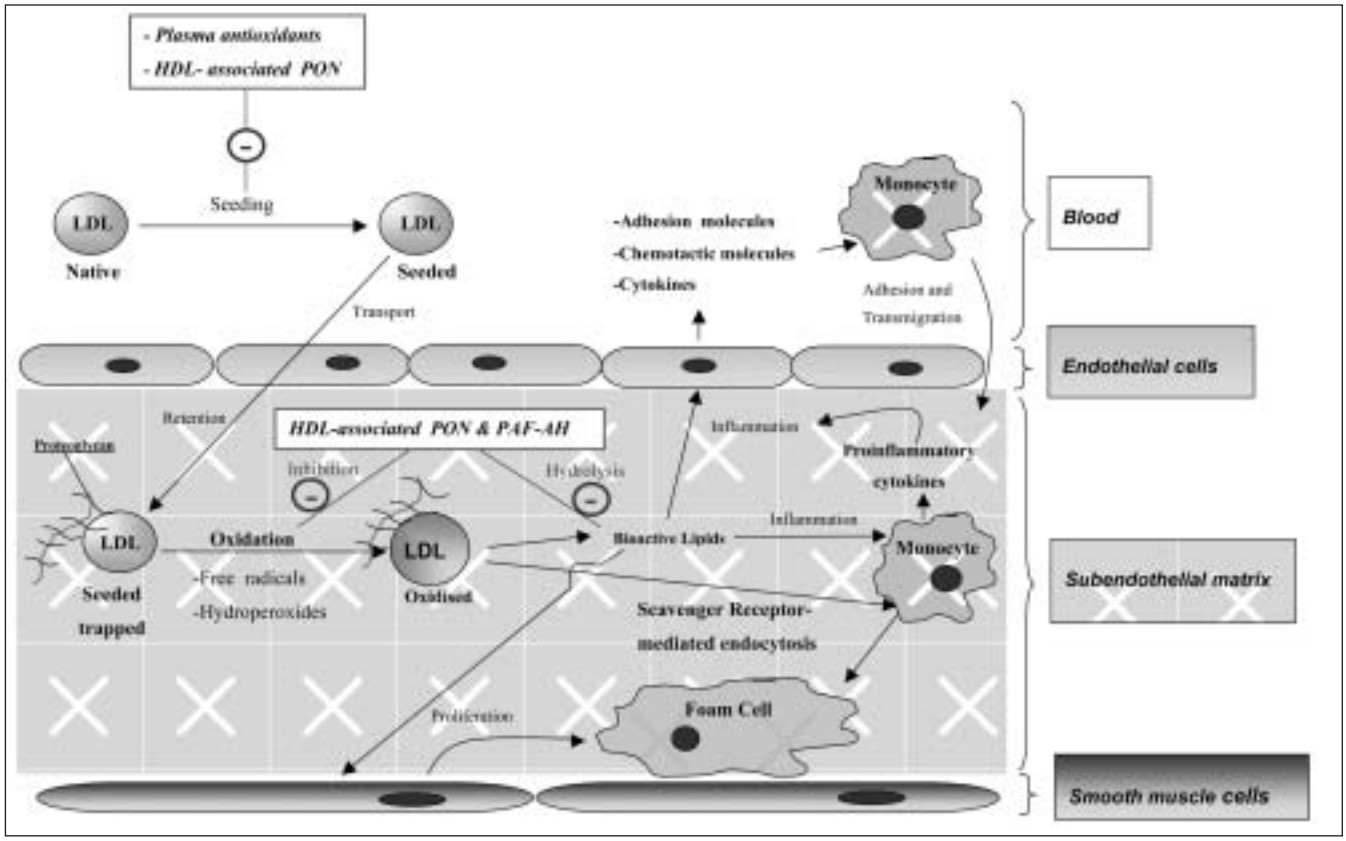

Figure 4. Hypothetical mechanisms by which paraoxonase (PON) and platelet activated factor acetylhydrolase (PAF-AH) protect against atherosclerosis.

may be vital for explaining the anti-atherogenic role of human apoA-I which, when expressed in transgenic mice, raises the activity of both paraoxonase and PAF-AH [253]. Other non-enzymatic properties directly mediated by apoA-I (but not by apoA-II or albumin) also block the steps that lead to LDL oxidative modification [254, 255]. HDL protection of LDL oxidative modification is altered in patients with coronary artery disease [254, 255].

At least some of these interactions between LDL and HDL may be mediated by the lipid transport proteins (CETP and LTP-2), proteins with an unknown function since the biological function of the exchange of lipids between lipoproteic particles is not evident [256]. It has been shown that HDL are the principal lipoprotein group in which cholesteryl esters with hydroperoxides accumulate [257]. At the same time, it is known that oxidised cholesteryl esters in HDL are cleared eight times more rapidly than HDL cholesteryl esters by cell cultures of hepatomas [258]. The SR-BI receptor seems to be involved in this clearance, which is not surprising if one considers that it belongs to the family of scavenger receptors [178, 231].

9. Therapeutic potential of increasing plasma HDL in atherosclerosis treatment and prevention.

Experiments with transgenic animals demonstrate that HDL containing apoA-I without apoA-II have greater anti-atherogenic force than HDL with both apoA-I and apoA-II [7, 64]. Moreover, overexpression of apoA-I in these animals is capable of protecting against atherosclerosis caused by various induced genetic defects (apoE deficiency or high levels of Lp(a), changes that supposedly increase atherosclerosis) [7, 8, 9, 10, 32]. Hence, one possible way to treat or prevent atherosclerosis is to administer apoA-I. The isolated injection of apoA-I in rabbits on a cholesterol-rich diet prevented lesion development, but did not reverse existing lesions [6].

Repeated HDL injection in rabbits on a cholesterol-rich diet does reduce atherosclerosis and even make it regress [5]. Short-term effects of intravenous apoA-I injection in humans have also been described [259]. The possibility of using apoA-I with mutations which seem to increase the beneficial effect of natural apoA-I, such as apoA-I Milano, also exists. There is considerable data on animal experimentation demonstrating the anti-atherogenic and anti-thrombotic power of apoA-I Milano [21-23], consistent with clinical and epidemiological data on the population where this mutation was detected. It is, therefore, plausible to believe that accumulated knowledge on the metabolism of HDL and their relationship with atherosclerosis, albeit incomplete, holds out a promise for future therapeutic interventions [260].

Another more conventional therapeutic alternative is based on the use of drugs to raise HDL cholesterol in plasma. Various clinical-epidemiological studies on patients treated with lipid-lowering drugs concluded that a 1% increment in HDL cholesterol concentration corresponded to a 3% reduction in the incidence of myocardial infarctions [2]. This has been demonstrated in secondary prevention of patients treated with gemfibrozil who showed decreased HDL cholesterol but normal LDL cholesterol [261]. However, this and other lipid-lowering treatments decrease plasma triglycerides; thus, in studies of this type with substances which only affect HDL, whether there is reduction, and to what degree, of atherothombotic cardiovascular events needs to be examined. One of the few drugs which act specifically by increasing HDL cholesterol is phenytoin, whose effect needs to be examined further in this type of study, especially in the course of treatment of patients with isolated hypoalphalipoproteinaemia [262]. Our group has treated different strains of mice susceptible to atherosclerosis with this drug, and although it does not reduce the area of atherosclerosis in apoE knock-out mice and LDL-receptor knock-out mice (Trocho *et al.*, unpublished data), it does improve the lipoprotein profile and decrease the atherosclerosis susceptibility of control mice, especially females [263], fed a high-fat diet.

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