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ROLE OF THE APOA5 GENE IN LIPID AND FAT-SOLUBLE VITAMIN METABOLISM
Montserrat Guardiola Guionnet
ISBN:978-84-691-9478-2/DL:T-19-2009

UNIVERSITAT ROVIRA I VIRGILI
Departament de Medicina i Cirurgia
Facultat de Medicina i Ciències de la Salut
Programa de doctorat en Nutrició i Metabolisme
Bienni 2003-2005

Role of the APOA5 gene in lipid and fat-soluble vitamin metabolism

Montse Guardiola Guionnet

2008

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Presentation & Justification

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Presentation and Justification

The work for the Doctoral Thesis that I am presenting has been carried-out in the Unitat de Recerca en Lípids i Arteriosclerosi, of the Departament de Medicina i Cirurgia of the Facultat de Medicina i Ciències de la Salut, of the Universitat Rovira i Virgili under the direction of Dr. Josep Ribalta.

The main interest of the Unitat de Recerca en Lípids i Arteriosclerosi has been the study of lipid metabolism, the mechanisms implicated in arteriosclerosis, its clinical presentation (cardiovascular diseases) and the associated risk factors, given the strong impact they have on the health of the population. Specifically, the line of investigation on the genetic predisposition to hypertriglyceridemia is based on the concept that the high levels of triglycerides are a key element in the development of cardiovascular diseases. In this sense, the focus of this thesis has been on the in-depth study the gene which has become the most important modulator of the levels of circulating triglycerides, the apolipoprotein A5 (APOA5). The gene was identified in 2001 and, at the time of commencing this PhD program in 2003, the mechanisms responsible for the strong impact that this gene has on triglycerides was not known. This thesis brings new knowledge on the role of the apo A-V. For this we used genetic markers that have enabled us to describe new associations of the gene with elements related to the metabolism of the triglycerides. We have used an animal model of arteriosclerosis to study the regulation of the gene by diet, and a cell model to confirm our hypothesis that apo A-V is also expressed in an organ involved in the processing of the triglycerides i.e. the intestine.

The thesis is divided into different sections. The Introduction contains an explanation of the themes that are necessary in order to put into perspective and to follow the course of the work conducted. The greater part of the knowledge on apo A-V is concentrated in the section dedicated to genetics in the Introduction, and receives less attention in the rest of the sections. The rationale has been that genetic studies are the ones that carry the major part of the information on this protein, the importance of which needed to be reflected in this thesis. Subsequently, the Hypotheses and Objectives justify the conduct of the different experimental studies performed, and which are presented in article format. This is followed by the General Discussion of the results obtained and the final Conclusions. The References section contains the details of the references cited in the previous sections. There is no Methodology section since the techniques

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used are routine in molecular biology and, further, are presented in detail in our published articles included in this thesis.

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Introduction

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1. Triglycerides and cardiovascular disease

Cardiovascular diseases are the principal cause of mortality and morbidity in Western and industrialized countries because their development are favored by factors strongly implanted in these societies, such as diet rich in fats and carbohydrates, stress, sedentary life-style and the tobacco habit, among others.

From among all the risk factors for cardiovascular disease (hypertension, thrombosis, obesity, and tobacco habit) possibly one of the most relevant is hyperlipidemia. Of special importance is the role placed by cholesterol since it is the lipid that accumulates in the atheromatous plaque and which finally leads to obstruction of the artery.

The concentrations of cholesterol transported by the low density lipoproteins (LDL) correlate directly with the prevalence and incidence of adverse cardiovascular events and death, and the therapies directed towards decreasing LDL cholesterol have demonstrated great efficacy in the prevention and the treatment of coronary disease (1). A key contribution to these notions derives from the Framingham study, which has as its clear objective the identification of the factors that promote cardiovascular disease and to follow the long-term developments in a large number of participants. The original cohort (1948) (2) was composed of 5209 men and women followed-up for 2 years. Over time this cohort widened with new generations of direct descendents of the original participants (The Framingham Offspring Study) (obtained from the website http://www.nhlbi.nih.gov/about/framingham/). Several years later (3), the study demonstrated that total cholesterol and the content of LDL are the principal inducers of arteriosclerosis in the greater part of the population, but that there are other important factors of cardiovascular disease risk. The conclusion from this study showed, for the first time that the low levels of high density lipoprotein (HDL) are also an important risk factor and that individuals, especially women, with high levels of triglyceride (TG) have a high relative risk of coronary disease. Other cardiovascular disease risk factors described in the study were hypertension, excess bodyweight, hyperglycemia, tobacco habit, lack of exercise and stress. This is relevant since it implies that the cholesterol-coronary artery disease relationship, although very solid, is fulfilled only in approximately 50% of cases of arteriosclerotic disease. This doctoral thesis is based, in great part, on the hypothesis that a significant proportion of the remaining 50% is explained by lipid alterations as well, but specifically related to hypertriglyceridemia (hyperTG).

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The association between the levels of TG and the risk of cardiovascular disease is somewhat confused.

Several epidemiological studies have shown that TG in the circulation are an independent risk factor for cardiovascular disease (4, 5, 6). A recent meta-analysis performed on >10,000 cases among a population >250,000 participants (7) indicated that the TG increases the risk of suffering an adverse cardiovascular event by 72% in addition to that carried by the traditional risk factors (cholesterol, glucose, hypertension, bodyweight, gender).

It is difficult to differentiate the risk induced directly by TG from that derived from the components of atherogenic dyslipemia (small dense LDL particles, elevated levels of apolipoprotein B, low levels of HDLc, remnant particles and postprandial lipemia) that characterize the moderately hypertriglyceridemic patient. Increasing evidence suggests that all the components of atherogenic dyslipemia can contribute to the development of atherosclerosis, and need to be considered in totality as a risk factor (8).

Plasma triglyceride concentration is a highly variable parameter that correlates with other lipids and lipoprotein parameters. As such, the specific contribution of TG to cardiovascular disease is consistently under-estimated in multivariate analysis (9).

Further, the increase in TG does not always imply development of coronary disease because the risk depends on the type of lipoprotein (intestinal or hepatic origin) in which the TG are transported. For example, while high levels of TG due to lipoprotein lipase deficiency are not atherogenic, moderate elevation of very low density lipoproteins (VLDL) are associated with a higher risk of cardiovascular disease (10).

All of this explains the declaration of the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) (11) which states that there are not sufficient data to confirm TG as an independent risk factor for cardiovascular disease.

However, a third of the population presents with pathologies that are related to hyperTG (diabetes mellitus type 2, obesity, FCHL,...) (12) and, as such, strengthens its possible role in atherosclerosis and justifies the study of factors that regulate its metabolism, especially its concentration in the circulation.

The apolipoprotein A-V gene has been considered a key gene in the control of TG levels, and its role in lipid metabolism is, in major part, the subject of the present thesis.

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2. Lipid metabolism

2.1. Exogenous lipid metabolism

The fats derived from the diet are mainly composed by TG and smaller quantities of cholesterol, cholesterol esters and phospholipids.

Digestion of fats occurs in the small intestine facilitated by bile salts which increase the surface of the lipid particles to enable the hydrolysis by lipolytic enzymes. The bile salts are synthesized in the liver from the lipids that are to be excreted, and are secreted via the bile into the duodenum where monoacylglycerols, free fatty acids, cholesterol and lipophosphoglycerols are formed which are absorbed in the microvilli of the absorptive cells of the intestine (the enterocytes) and re-esterified *de novo* forming TG, phospholipids and cholesterol esters in the endoplasmic reticulum.

Lipids are insoluble and to be transported in plasma they need to be carried as LIPOPROTEINS which are macromolecules stable in aqueous medium. The hydrophobic nucleus transports non-polar lipids (TG and esterified cholesterol) and is enveloped within a hydrophilic surface containing amphipathic lipids (phospholipids and non-esterified cholesterol), as shown schematically in Figure 1.

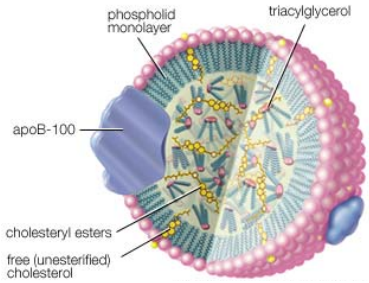

Figure 1. Lipoprotein molecule organization

Classically, the lipoproteins have been classified on the basis of their electrophoretic mobility in a basic pH medium; a method that distinguishes three mobile bands (corresponding to the mobility of β-globulin, α2-globulin or α1-globulin) and one static band. An alternative classification is based on the hydrated density of the lipoprotein which enables the lipoprotein to be separated by ultracentrifugation; the densities being a function of the lipid and protein contents (summarized in Tables 1 and 2).

The lipoproteins have four main functions: transport of exogenous TG, transport of endogenous TG, transport of cholesterol, and the removal of excess cholesterol. Apart from the lipids, the lipoproteins enable the transport of molecules derived from the diet such as liposoluble vitamins (vitamins A, D, E and K), among which vitamin E is of considerable because, apart from its known antioxidant role (an important process in the evolution of arteriosclerosis), its metabolism proceeds in parallel to that of the

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triglyceride-rich lipoproteins (TRL). The protein component of the lipoproteins are formed, principally, by the APOLIPOPROTEINS which are involved in the assembly of the lipoproteins, in stabilizing their structure, acting as co-activators of enzymes or acting as ligands of receptors for their cellular uptake. The principal apoproteins involved in lipid metabolism are summarized in Table 2.

	CHYLO	VLDL	IDL	LDL	HDL
PHYSICAL PROPERTIES					
Electrophoretic mobility	Origin	Pre-β	Pre-β	β	α
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Molecular weight (Da)	$4x10^6$	$10\text{-}80x10^6$	$5\text{-}10x10^6$	$2\text{-}3x10^6$	$0.2\text{-}0.4x10^6$
Density (g/ml)	<0.93	0.93-1.006	1.006-1.019	1.019-1.063	1.063-1.210
COMPOSITION					
Protein (%)	2	10	11	23	55
Phospholipids (%)	9	18	25	20	24
Free cholesterol (%)	1	7	10	8	2
Cholesterol ester (%)	3	12	32	37	15
TG (%)	85	50	25	10	4

Table 1. Characteristics of the lipoproteins

Apolipoprotein	Molecular weight (kDa)	Associated with
Apo A-I	28	Chylo, HDL
Apo A-II	17	Chylo, HDL
Apo A-IV	44	Chylo, HDL
Apo A-V	40	Chylo, VLDL, HDL
Apo B48	240	Chylo
Apo B100	513	VLDL, IDL, LDL
Apo C-I	7	Chylo, VLDL, IDL, HDL
Apo C-II	9	Chylo, VLDL, IDL, HDL
Apo C-III	9	Chylo, VLDL, IDL, HDL
Apo E	34	Chylo, VLDL, IDL, HDL

Table 2. Characteristics of the apolipoproteins

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Chylomicron. Intestinal cells synthesize the lipoproteins responsible for transporting in the blood the lipids from the diet, mainly, TG, to the liver and other tissues, and are termed CHYLOMICRONS. Analytically, they are characterized by not having electrophoretic mobility in basic pH medium. They are the largest lipoproteins with 85% of their content in the form of TG, and have the lowest density (<0.93 g/mL) (Table 1).

The process of intestinal lipoprotein synthesis (13) can be divided into three stages:

- Assembly of the primordial lipoprotein
- Formation of lipid droplets
- Expansion of the nucleus of the particle

The process can be summarized as follows. The absorbed lipids, mainly TG, begin to associate with the key apoprotein in this process, apoprotein B48, while its translation is taking place in the ribosomes.

Apolipoprotein B is a large protein (515 kDa) composed of a series of organized domains: NH_2 / α-helix$_1$ / lamina-β$_1$ / α-helix$_2$ / lamina-β$_2$ / α-helix$_3$ / COOH. The α-helix$_1$ domain is homologous to lipovitellin which is a protein that participates in the transport of lipids between the liver and the oocyte in oviparous animals. The β-laminas are thought to be the direct and irreversible interaction with lipids of the nucleus of the lipoprotein. Apo B exists in two forms, apo B100 and apo B48. By a process of alternative splicing that converts a glutamin to a stop codon, the resultant apo B48 corresponds to 48% of the n-terminal part of apo B100. The majority of publications (14) show that, in humans, apo B100 is only present in the lipoproteins derived from hepatic synthesis (VLDL and its remnants) and that apo B48 is only present in the lipoprotein of intestinal origin (chylomicrons and its remnants), although there are some studies that describe the presence of B100 in the intestine as well (15). There is only one molecule of apo B per lipoprotein particle and, hence, measurement of plasma levels of apo B reflects the number of circulating apo B-containing lipoprotein particles.

MTP is another key protein in the process of synthesis of TRL. It is present in the endoplasmic reticulum and, as well, has a n-terminal domain homologous to lipovitallin. It enables nascent apo B to become enriched in lipids as a result of transfer of polar and neutral lipids from the membrane of the endoplasmic reticulum. Alterations in the domains not only of apo B but also MTP are described in subjects with abetalipoproteinemia (i.e. those pathologies in which apo B is not synthesized) (16).

Apo B that is not lipid associated is degraded predominantly via the ubiquitin/proteasome system. There are other proteolytic systems that can act on apo

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B in various stages of the secretion pathway e.g. the immediate uptake of recently-synthesised VLDL by the LDL receptor, or pre-secretory degradation (17).

While apo B is being translated by the ribosomes, it becomes enriched with lipids via the MTP. For the formation of the lipoprotein particle to be initiated, there needs to be a minimum length containing the first two functional domains of the apo B. As a result of the increasing length of this apoprotein the TG:phospholipid ratio is also increased, forming the TG-rich nucleus. This complex is then directed towards the Golgi apparatus where the phospholipids and apolipoprotein A are added. Of special note at this stage is the role of apo A-IV in modulating the assembly of the chylomicrons (18). The final result is a nascent chylomicron that is released into the lymphatic system connected to the general circulation via the thoracic duct (Figure 2).

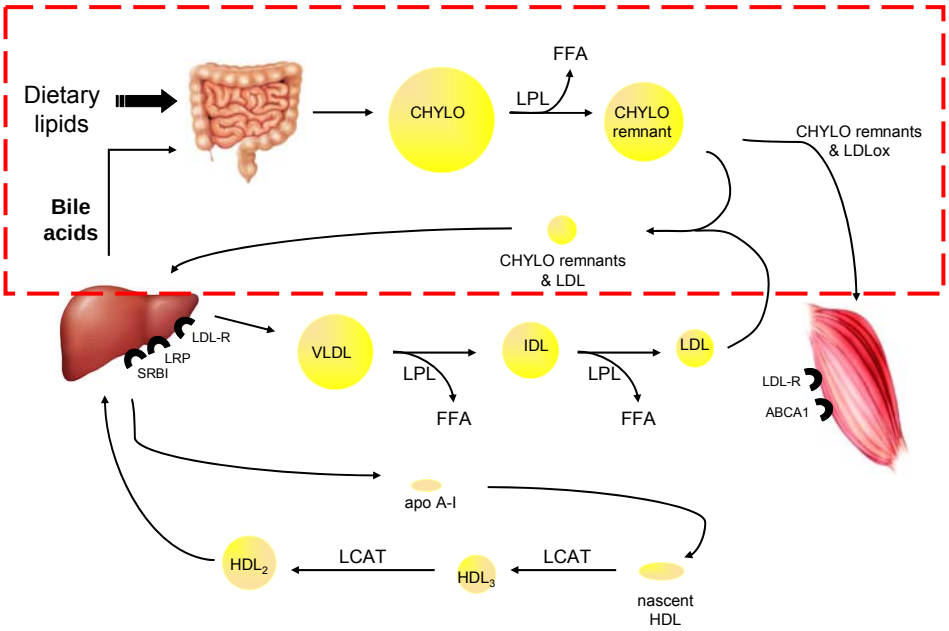

Figure 2. Overall scheme of the metabolism of human lipoproteins. The stages of metabolism of exogenous lipids are contained within the box outlined in red.

As summarized in Table 1, the nascent chylomicrons are composed, principally, of TG and smaller quantities of phospholipids, free and esterified cholesterol, with the protein component composed of apo B48, apo A-I, apo A-II and apo A-IV. This makes the nascent chylomicrons good acceptors of free cholesterol derived from other lipoproteins. This process of interchange takes place in the general circulation and is mediated by the lipid transfer proteins (19), phospholipid transfer protein (PLTP) and the cholesterol ester transfer protein (CETP) The interchange involves the apo As, apo Cs and apo Es between the nascent chylomicrons and HDL, enabling the nascent

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chylomicrons to mature and to become a substrate for the action of lipoprotein lipase (LPL). This process of interchange between lipoproteins is important in maintaining the levels of HDL; a low activity of PLTP markedly decreases the lipids and apoproteins of HDL.

CETP is a hydrophobic glycoprotein secreted principally by the liver and circulates in plasma bound mainly to HDL. It facilitates the redistribution among circulating lipoproteins of cholesterol esters and TG and, to a lesser extent, phospholipids. This process results in an equilibration of lipids among the lipoproteins and, overall, the CETP transfers cholesterol esters from HDL to the TRL and LDL, and transfers TG from the TRL and LDL to HDL. As such, the transfer mediated by CETP indirectly promotes the elimination of cholesterol esters from the circulation via the liver (20). Because of its key role in the metabolism of HDL, alterations can have an impact on the susceptibility to arteriosclerosis, as demonstrated in cases of human CETP deficiency (21). This explains the interest in conducting clinical trials assessing the effect of CETP inhibitors (including the drug torcetrapib) on cardiovascular disease (22). However, the clinical trial conducted in 2006 showed an excessive mortality in the group treated with torcetrapib in combination with a statin (23) and the drug was withdrawn from the market.

The process of interchange enables the TRL to acquire apolipoproteins essential for the hydrolysis of the TG, such as C-II which is the activator necessary for the enzyme LPL, and apo C-III which is the inhibitor of its activity. Although apo C-II is essential for the function of LPL, apo C-III is very important because it fulfills a double function of inhibiting the LPL and of modulating the uptake of lipoproteins by interrupting the recognition of apo E by the hepatic receptors (24, 25). As such, apo C-III is associated with the catabolism of the triglyceride-rich lipoproteins.

LPL is a glycoprotein synthesized in heart, muscle and adipose tissue. For its physiological function in the metabolism of the TG it is secreted in the vascular endothelium bound to the proteoglycan heparin sulfate. It functions as a homodimer hydrolyzing the TG of the circulating lipoproteins that contain apo C-II (26), a process that is stimulated by apo A-IV (see Genetics of A-IV section).

As the chylomicrons circulate they encounter the active LPL bound to the endothelium certain tissue (adipose tissue, muscle, mammary glands…). The TG content is hydrolyzed resulting in the liberation of the fatty acids which are taken-up by the tissues for storage or used to provide energy, and the glycerol part of the molecule is integrated into the gluconeogenesis pathway. LPL is the key in the process of receptor mediated elimination of lipoproteins (27).

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The mean life-span of chylomicrons in circulation is approximately 1 hour (although by the time they appear in the blood this can be up to 4 hours). After 80-90% of the TG has been hydrolyzed and apo Cs and apo As transferred to HDL, the chylomicrons become reduced in size, denser and containing mainly cholesterol and apo E, and are termed chylomicron remnants.

Apo E is a key protein in the catabolism of the triglyceride-rich lipoproteins (chylomicrons and VLDL) because it is the apoprotein responsible for recognition by the specific receptors (the LDL receptor; LDL-R) and the protein related to the LDL-R (LRP) in the liver and peripheral cells that facilitate the elimination of the remnants of the TRL from the circulation.

Apo E is synthesized mainly in the liver, but in lesser amounts in other tissue such as the intestine. In humans the APOE gene is polymorphic and presents as 3 alleles E2, E3 and E4 giving rise to 6 possible genotypes (E2/E2, E3/E3, E4/E4; E2/E3, E2/E4, E3/E4). The 3 alleles are distinguished by the presence/absence of Cys and Arg amino acids at the polymorphic sites, their affinity for the LDL-R, and their consequent binding with lipids and lipoproteins (28):

- Allele E3 (Cys112 and Arg158) is the most frequent (70-85%) in the general population
- Allele E4 (Arg112 and Arg158) is present at a frequency of 12-18% in the general population. It has high affinity for the LDL-R and, compared to E3, is associated with higher circulating levels of LDLc and TG.
- Allele E2 (Cys112 and Cys158) is present at a frequency of 3-12% in the general population. It has the lowest affinity for the LDL-R and, relative to E3, is associated with the low levels of LDLc and high levels of TG.

In the liver there are receptors on the cell surface such as those of the LDL-R subfamily which enable the internalization of the remnant particles of the TRL which have become depleted of their cholesterol content and which will be eliminated or metabolized. The apolipoproteins (such as apo E and apo B100) are the ligands for these receptors (29). As we will see later, apo A-V can recognize and stimulate the elimination of these particles.

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2.2. Endogenous lipid metabolism

There are fatty acids of diverse origins in the liver:

- Derived from the circulation by uptake of the remnant particles, or HDL
- Taken-up from the circulation bound to albumin, derived from the lipolysis in the adipose tissue (predominantly fasting, with fat-rich diet or in pathological states such as diabetes mellitus)
- Synthesized *de novo* from acetyl-CoA derived from glucose, lactose or alanine (predominantly in the post-prandial state).

VLDL. The liver secretes the TG of endogenous origin to form part of the VLDL. Analytically, they are characterized by electrophoretic mobility in basic pH equivalent to that of α2-globulin. TG accounts for 50% of its content the hydrated density separated by gradient ultracentrifugation is in the range of 0.93-1.006 g/mL. The process of assembly of these particles is very similar to that of the chylomicrons (see section 2.1) and is summarized in Figure 3.

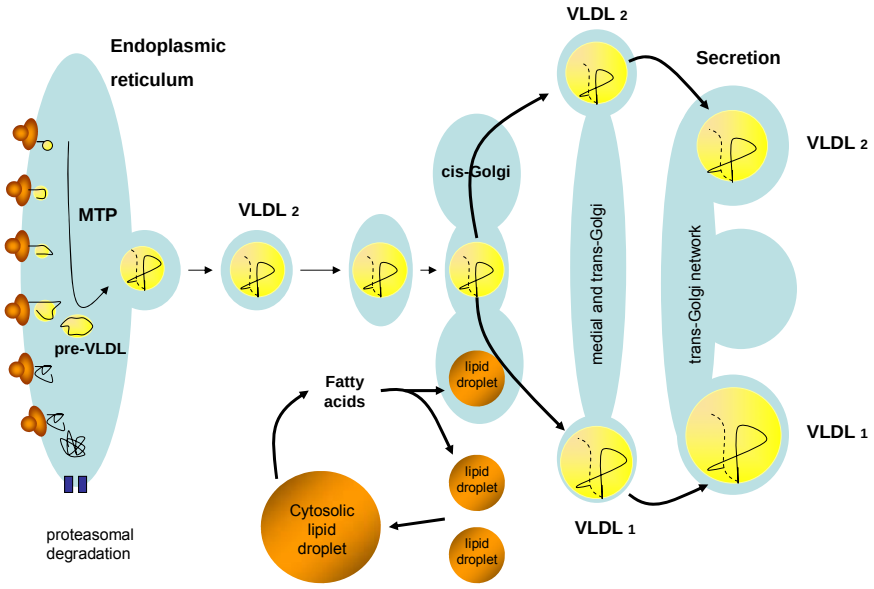

Figure 3. The intracellular assembly of VLDL lipoproteins. Adapted from (30). The process begins in the endoplasmic reticulum with the synthesis of apo B100 that interacts with the MTP which will lipidate to form the pre-VLDL (if not, it will be degraded by the proteosomal complex). The pre-VLDL is converted to $VLDL_2$ which, in the Golgi apparatus, can be transformed to $VLDL_1$ if the intra-cellular pool is high.

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Nascent VLDL contains mainly TG, albeit somewhat less than the chylomicrons (see Table 1), and, as such, are smaller than the chylomicrons. They contain apolipoproteins E, C-I and B100 and, unlike the chylomicrons, do not have apo B48 (see Table 2). Once in the circulation (Figure 4) they begin to mature by interacting with other lipoproteins such as HDL with which it can interchange cholesterol and apolipoproteins, involving, above all, the transport proteins such as CETP. When VLDL has become enriched with apo E and apo Cs, the LPL anchored to the capillaries hydrolyzes the TG from the apolar nucleus, the particle begins to lose part of the surface together with apo C and phospholipids are transferred to HDL.

IDL. By the action of LPL, the VLDL is transformed to VLDL remnants or IDL which are smaller and denser. More cholesterol is transported and the main apoproteins are apo B100 and apo E. The mean life-span of the VLDL particle in circulation is 1-3 hours. One part (approximately 50%) of the IDL particles formed are taken-up by the liver and eliminated from the circulation or are transformed to LDL as a result of the lipid transfer proteins which, in the circulation, facilitate the interchange of their components with HDL. The rest of the IDL particles are transformed to LDL by the action of hepatic lipase.

Hepatic lipase is a lipolytic enzyme which has phospholipids as its principal substrate. It participates very actively in the remodeling of HDL and LDL. It is anchored in the liver and hydrolyses TG that is subsequently internalized in the liver. Apart from the hydrolytic activity, it also acts as a ligand for the uptake of lipoproteins via the cell surface receptors and proteoglycans (31).

LDL. These particles are, essentially, of plasma origin but can be synthesized in the liver, as well (32,33). They are small in size and are composed mainly of cholesterol esters and one molecule of apo B100. They are characterized by having β-globulin mobility in basic pH, and separated by ultracentrifugation in the density rages of 1.019-1.063 g/ml. LDL are the principal method of transport of the greater part of the cholesterol in the circulation. The mean life-span of LDL is 2-3 days. The process of catabolism of LDL takes place principally in the liver (excess cholesterol being excreted together with bile) but other lipid components are catabolized via the intestine or are used in steroidogenesis (for the production of sex hormones), by macrophages and other tissues of the organism for the formation of membranes.

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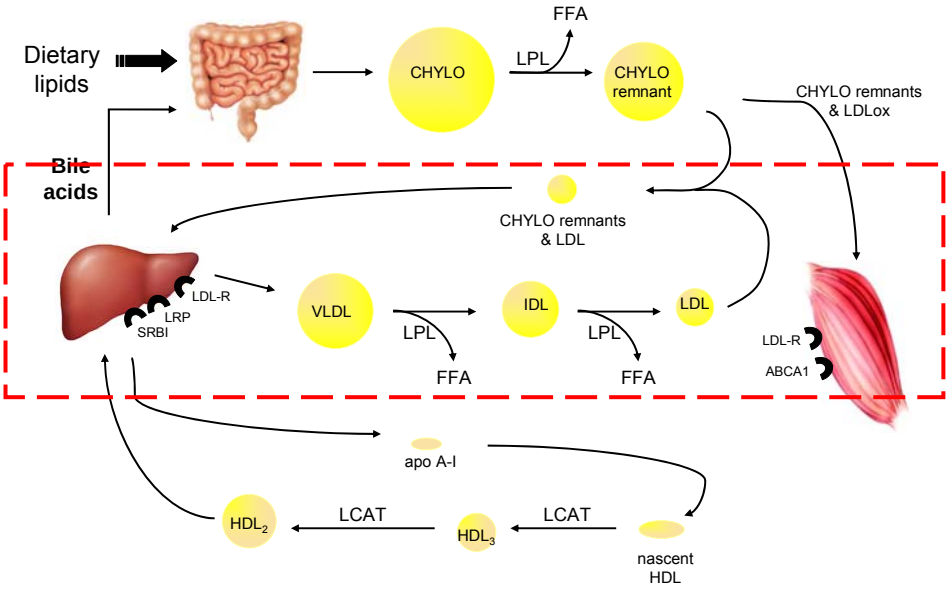

Figure 4. Overall scheme of human lipoprotein metabolism. The metabolism of endogenous lipids is described within the box outlined in red

LDL particles are eliminated from the circulation via the LDL receptor (LDL-R) which is a transmembrane glycoprotein of 839 amino acids located on the cell surface. Control of the levels of plasma cholesterol is via the uptake (by a process of endocytosis) and catabolism of the plasma lipoproteins containing apo B100 (present in LDL) and apo E (present in the chylomicron and VLDL remnants). The receptor, together with the lipoprotein particle, is internalized by endocytosis and, once completed, the receptor is recycled to the plasma membrane. As such, its transcriptional expression is regulated by the levels of intra-cellular free cholesterol. The defect in LDL-R results in familial hypercholesterolemia (FH); the heterozygotes of this condition express half the number of functional LDL-R (1 in 500 individuals) and the homozygous individuals express very few or no functional cell surface receptors (1 in 10^6 individuals). Patients with FH have double the levels of LDL cholesterol in infancy relative to normal values, and develop early coronary disease (at a mean age of 45 years, in males) (34).

LDL and the development of arteriosclerosis

Cholesterol transported by LDL has a key role in the development of coronary ischemia, as has been demonstrated by several epidemiological studies. Briefly, in conditions of excess circulating cholesterol, activation of the endothelium of the large and medium sized arteries occurs and the lipoproteins are able to infiltrate the arterial wall and to accumulate in the extra-cellular matrix. Oxidation of these LDL liberate

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bioactive phospholipids that activate the endothelial cells to expressing adhesion molecules (such as vascular cell adhesion molecule; VCAM-1) which attract various blood cells such as monocytes which penetrate and differentiate into macrophages that express cell surface scavenger and toll-like receptors. The macrophages play a key role in the development of atherosclerosis.

The scavenger receptors recognize LDL modified by acetylation or oxidation and, unlike the normal cell which has intra-cellular cholesterol feedback control of LDL-R synthesis it is this lack of intra-cellular feed-back that allows the macrophages to continue to accumulate modified cholesterol and to become foam cells which, deposit their lipid content in the sub-endothelial space and contribute to the development of the atherosclerotic plaque.

The toll-like receptors do not undergo endocytosis. Instead they initiate a cascade of signals that result in the macrophage being activated to liberate, among other molecules, nitric oxide (a reactive species of oxygen) and to secrete proteolytic enzymes that degrade the matrix resulting in a destabilization of the plaques which increases the risk of their rupture and of thrombosis.

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2.3. Reverse cholesterol transport

We have seen how the endogenous cholesterol and the exogenous cholesterol of the diet are distributed throughout the body. The liver and the steroid hormone producing organs can utilize the cholesterol for the synthesis of cellular components, but the rest of the tissue of the organism cannot degrade cholesterol and, as such, need to dispense with the excess cholesterol since, as we have seen in section 2.2, this can lead to adversely affecting the physiological function of the cell.

HDL. The reverse transport of cholesterol is mediated by HDL (depicted in Figure 5). HDL particles are characterized as having α1-globulin electrophoretic mobility and with high density (1.063-1.210 g/ml) (Table 1). These lipoproteins are very heterogeneous and contain different sub-fractions which differ in size, density and composition of apolipoproteins and lipids. Nascent HDL are discoidal in structure (pre-β-HDL) and derive from synthesis in the liver and the intestine or from the liberation of A-I from TRL following processing by LPL (35).

Apolipoprotein A-I is synthesized principally in the liver, and to a lesser extent in the small intestine. It is abundant in plasma (1.0-1.5 mg/dl). It is the principal protein component of HDL and its concentration reflects the concentration of circulating HDL. It plays an important role in cellular homeostasis of cholesterol. High levels of apo A-I are associated with a reduced risk of cardiovascular disease, and epidemiological studies sugges that the levels of apo A-I can be a better marker of cardiovascular disease risk than the levels of total cholesterol, or of the lipid content of the lipoproteins (36-39).

Reverse cholesterol transport involves the process of capture by HDL of excess cholesterol in peripheral cells, and the transport to the liver where the cholesterol is eliminated in bile. Hence, the cholesterol transported by HDL has been considered as a protective factor against cardiovascular disease.

The process can be divided into three stages:

- Efflux of cholesterol from the cells
- Esterification of cholesterol
- Metabolic destination of the cholesterol esters formed

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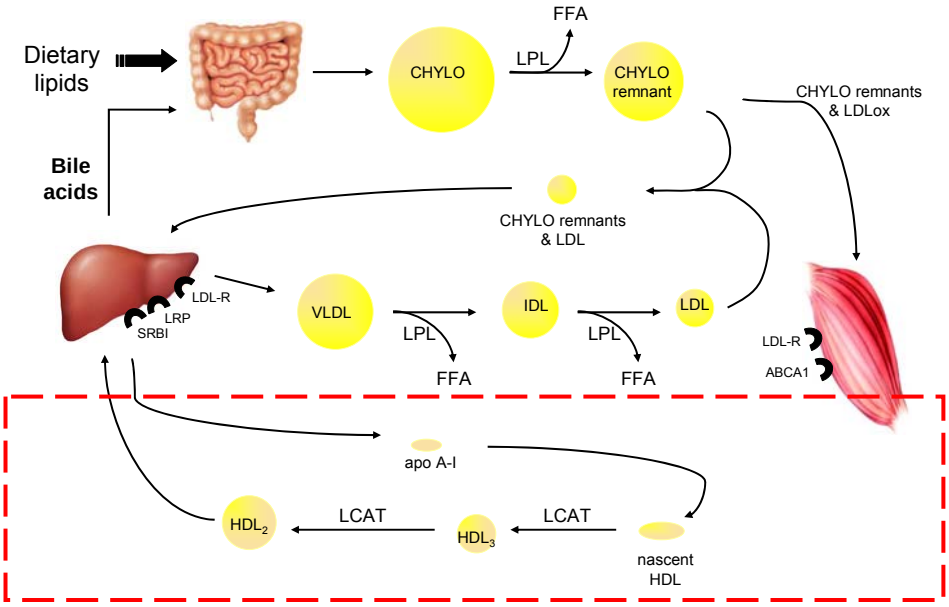

Figure 5. Overall scheme of human lipoprotein metabolism. Reverse cholesterol transport is described within the box outlined in red

Nascent HDL particles have a high capacity to capture cholesterol from peripheral tissue (such as arteries) and from macrophages, and which explains the protective role against the development of atherosclerosis. The mechanisms responsible for the transfer of the cholesterol from the cells to HDL have not been fully elucidated. Two hypotheses have been proposed:

- By receptor-independent diffusion in which HDL with apo A-I, as well as HDL with apo A-I plus apo A-II, may participate
- A receptor-dependent process that requires specific acceptors such as apolipoproteins A-I, A-II, A-IV, E and C, interacting with pre-β-HDL particles. In this case the lipidation of nascent HDL takes place as a result of the efflux of cholesterol mediated by the membrane transporter ABCA1 (ATP-binding cassette A1) (40). Mutations in the ABCA1 gene produce Tangier disease.

Currently, it is accepted that both types of processes could co-exist and that the predominance of one of the other process depends on the type of cell involved.

Free cholesterol that is captured is rapidly esterified by LCAT (lecithin: cholesterol acyl-transferase) which is an enzyme bound to the surface of HDL and activated by apo A-I. The esterified cholesterol passes to the nucleus of the particle. With this process of esterification, the HDL particle begins to assume the spherical form, becomes much larger and transforms into HDL_3 and subsequently into HDL_2. These particles can

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interact with the protein ABC-G1 and promote a level of intra-cellular cholesterol greater than that of macrophages (41).

The destination of the cholesterol esters in HDL varies. HDL can be recognized directly by hepatic receptors, SR-BI, or by apo E receptors. Once internalized, the cholesterol can be used for the assembly of lipoproteins, as substrates for the synthesis of bile acids, or secreted directly into the bile. Since there is no uptake of the particle, the HDL can be recycled to the circulation to begin the process over again. Another possibility is that HDL particles are captured in entirety by the LDL related receptor (LRP) or, in an indirect manner, the cholesterol esters contained in the HDL are transferred to the TRL via the lipid transfer proteins such as PLTP and CETP.

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3. Dyslipidemia: focus on hypertriglyceridemia

3.1. Hypertriglyceridemia

Hypertriglyceridemia is defined as the presence of elevated levels of TG in fasting plasma, and is associated with diabetes mellitus, abdominal obesity, insulin resistance, or secondary to treatment with some pharmacological agents. The most recent recommendations place the upper limit of plasma TG values at 1.7 mmol/L (150 mg/dL) since that above this level the small dense LDL particles that are highly atherogenic begin to increase (42). These values can be around 20 mmol/L in the more severe cases of hypertriglyceridemia.

HyperTG is the consequence of disequilibrium in the process of production of lipoproteins rich in TG in the liver and/or intestine, and of their hydrolysis in the circulation.

Several grades of hypertriglyceridemia between moderate and severe have been described and can fluctuate even in the same individual, depending on the metabolic status. As shown in Figure 6, there is an inverse relationship between the grade of hyperTG and its presence in the general population. Severe hyperTG with considerable elevations of circulating TG can occur infrequently, while moderate hyperTG is more frequent. As such, primary hyperTG has a much lower influence on the incidence of arteriosclerosis that secondary hyperTG.

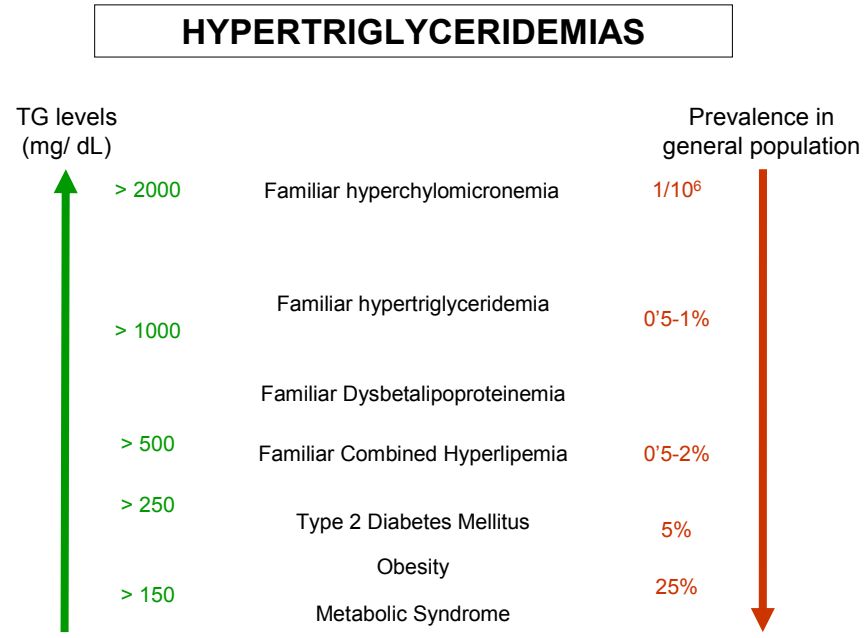

Figure 6. Inverse relationship between different inherited forms of hypertriglyceridemia and their prevalence in the general population

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The hypertriglyceridemias are classified as primary and secondary according to the cause. The primary hypertriglyceridemias are of genetic origin, and are more severe than the secondary hypertriglyceridemias which are due to external factors.

3.1.1. Primary hypertriglyceridemias

FAMILIAL COMBINED HYPERLIPIDEMIA

This disease is a basic alteration in the metabolism of TG, and is the defect of lipid metabolism of considerable clinical importance because it is the most common genetic form of hyperlipidemia. It is present in 1-6% of individuals in the populations of developed countries and in 20% of the survivors of myocardial infarction. As such, it considered as a significant risk factor in the development of cardiovascular disease (43).

The traditional diagnostic criteria are based on the isolated hypercholesterolemia and hypertriglyceridemia or combined. Over the past several years and after several reviews of the evidence, apo B has consistently remained as a parameter that is characteristic of this alteration, together with the overproduction of VLDL and the presence of small dense LDL particles (sdLDL) (44).

There have been 2 strategies employed in the process of identifying the underlying genetic alterations and the genetic markers for the diagnosis of FCH: linkage studies and association studies which enable conclusions to be drawn regarding a complex disease that appears not to have a common genetic basis. This is reflected in the multiple lipoprotein phenotypes that exist in the families of affected patient and, as well, in the same individual over time. In linkage studies, FCHL has been strongly associated with the human chromosome 1 (45) where of note is the upstream transcription factor 1 (USF1) which regulates the expression of the genes involved in the metabolism of glucose and of lipids. Further, this chromosome also contains the gene for APOA2 which has been associated with high levels of TG as a result of accumulation of large VLDL particles, and CRABP2 (46, 47) which we recently described as being associated with elevated levels of plasma cholesterol. Several association studies have identified gene modulators of hyperlipemia in these FCHL patients and of note is the region of the gene complex coding for the apoliproteins A1/ C3/ A4/ A5 located on human chromosome 11, and specifically the APOC3 and APOA5 genes which have been shown to exercise an influence on the hyperTG in FCHL patients (48-51).

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FAMILIAL DYSBETALIPOPROTEINEMIA

Present in 1/2000-5000 persons, this is a mixed hyperlipidemia with elevations of similar magnitude not only of cholesterol but also TG, secondary to the increase in the β-VLDL (which are remnants of the chylomicrons of intestinal origin) and of the VLDL of hepatic origin. The accumulation of remnants is a consequence of the homozygous defect in the APOE gene which is responsible for the uptake of the remnants by the LDL-R. It is only present in carriers of the APO E2/E2 genotype, or rare mutations such as that which produces the G127D change. E2/E2 homozygosity is a necessary but not sufficient cause of the disease since only 3% of these individuals develop the disease (52). The co-adjuvant factor is, in many cases, another genetic defect such as mutations in the APOA5 gene (53), or some pharmacological treatment such as with retinoids. The patients develop xanthomas and premature coronary disease.

FAMILIAL HYPERTRIGLYCERIDEMIA

With a prevalence of 5-10%, this disease is characterized by the presence of elevated VLDL triglycerides alone. The levels of cholesterol are normal, or only slightly elevated. Although the underlying causes have not been fully elucidated, it is found to be associated with obesity, insulin resistance, diabetes, hypertension and hyperuricemia. It represents a heterogeneous disorder with a phenotype that is highly influenced by environmental factors, as has been demonstrated in patients heterozygous for the Q139X mutation of the APOA5 gene who are predisposed to hyperTG and, probably, in combination with other genetic or pathologic factors such as obesity or diabetes (54).

A genome-wide scan conducted in 26 families with familial hyperTG (55) identified chromosomal zones that strongly influence parameters linked to alterations in the metabolism of TG (as we will see later). Other recently-identified influences are chromosome 6 which appears to be associated with the size of LDL particles (a candidate gene on this chromosome could be superoxide dismutase, which is of considerable importance given that the small dense LDL particles have a higher susceptibility to oxidation than the larger LDL particles). Chromosome 15 shows some association with TG levels (a candidate gene could be that for HL) and a weaker association between regions of chromosome 18 and levels of HDL cholesterol have been noted (no obvious genes related to the metabolism of HDL metabolism have been encountered on this chromosome).

FAMILIAL CHYLOMICRONEMIA

This is a very rare condition characterized by extremely high levels of TG since birth

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(1000-10000 mg/dL) (Figure 7). It is characterized by the presence of chylomicrons even after 12-14 hours postprandial phase. It is due to a defect in the LPL or, rarely, to a defect in the enzyme's activator (APOC2) although one patient who did not have either of these genetic defects was described as having severe hyperchylomicronemia due to a mutation (Q145X) in the APOA5 gene (56). The patients develop xanthomas and recurrent pancreatitis, but usually do not develop atherosclerosis although this has been described in one case (57).

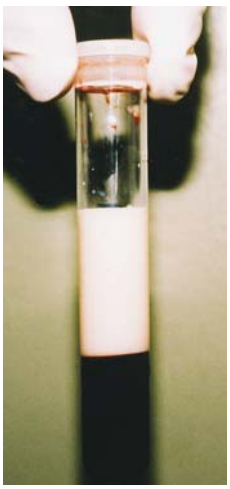

Figure 7. Lipemic serum

3.1.2. Secondary hypertriglyceridemias

Hypertriglyceridemia is a lipid alteration that is characteristic of "an atherogenic life-style" which implies an unhealthy diet, lack of exercise and is associated with obesity, insulin resistance, long-term diabetes mellitus, and cardiovascular disease.

OBESITY

Obesity is the most prevalent metabolic disease in developed countries, and continues to increase alarmingly to epidemic proportions. It carries with it a high mortality from cardiovascular disease, resulting directly or indirectly from its association with multiple risk factors.

Overweight is defined as a body mass index (BMI) value between 25 and 30 kg/m^2 and, in the case of obesity, the BMI is >30 kg/m^2. The concept of visceral obesity, defined clinically as a waist circumference of >88 cm in females and >102 in males, is of considerable usefulness because is related to metabolic and cardiovascular alterations associated with the metabolic syndrome or insulin resistance. As such, if in combination these factors determine the levels of TG (concept termed waist hyperTG) this measurement provides a cheap tool for the identification of patients with an elevated risk of cardiovascular disease (as will be explained later).

Adipose tissue is an organ for reserve storage and mobilization of fats and, currently, interest is increasing with respect to its function as an endocrine organ since it liberates several cytokines such as TNF-α and IL-6 known as pro-inflammatory molecules (58). Fats are stored in the form of TG which are derived from circulating chylomicrons or VLDL. The TG are hydrolyzed by LPL bound to the capillaries of the adipose tissue.

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The resultant free fatty acids (FFA) are taken up by the cells, internalized and re-esterified. Patients with a deficiency in LPL also accumulate TG in the adipose tissue (59) and, as such, alternative routes such as the acylation stimulating protein need to be postulated (60).

The defect in the metabolism of the FFA contributes to the insulin resistant status of the individuals with visceral obesity. The lack of response to control by insulin leads to visceral tissue adipocyte hypertrophy undergoing a high grade of lipolysis, resulting in a high flow of FFA to the liver. This alters the hepatic metabolism resulting in an increase in the production of glucose and the secretion of TG. Insulin resistance in the liver is associated with a lower control of apo B degradation, and an increased production of TRL.

The concept of waist hyperTG (61) suggests the simultaneous use of waist circumference and the levels of fasting TG to identify individuals characterized by their atherogenic triad (presence of small dense LDL particles, elevated levels of apo B, and hyperinsulinemia), which results in an elevated cardiovascular disease risk.

DIABETES MELLITUS TYPE 2

This is the most common type of diabetes in developed countries. It is strongly linked with obesity (80% of individuals with DM2 are obese) (62). Its incidence is progressively increasing, and it is forecast by the WHO that it will become an epidemic. It only develops in middle-age, although the incidence in young people is increasing. Adults with DM2 have a 2- to 3-fold higher risk of dying from cardiovascular disease than those adults without DM2 (63). This implies that, in the calculation of cardiovascular disease risk, DM2 is equivalent to having suffered an episode of ischemic cardiopathy.

DM2 is characterized by insulin resistance and decrease in insulin secretion. The resistance to insulin takes place when the organism cannot use the insulin efficiently. For example, the liver and muscle have a decrease in the biological effects of response to insulin (which essentially maintains glucose levels in the blood, stimulates lipogenesis and decreases lipolysis). In an attempt to balance the circulating levels of glucose, the pancreas secretes increasing amounts of insulin and, gradually, the pancreatic cells become deficient, and begin to decrease in number. Hence, the individuals who are insulin resistant have fasting hyperglycemia and hyperinsulinemia and, at the same time, have alterations in liver function and concentrations of fatty acids in the circulation.

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Diabetic patients, or those with diabetic dyslipemia, show disequilibrium in the lipid parameters in the blood, essentially of total cholesterol. This is accompanied by an increase in LDLc in females and hypertriglyceridemia together with a decrease in the levels of HDL cholesterol as well as the presence of small dense LDL particles. The hypertriglyceridemia in DM2 is due to an increase in the plasma concentration of VLDL, with or without chylomicronemia; a lower activity of TG hydrolysis by LPL; an increase in lipid interchange via CETP; and a lower flow of FFA to the liver.

In summary, DM2 results in a lipid profile that causes arteriosclerosis and premature cardiovascular disease.

RENAL DISEASE

Chronic renal insufficiency presents with considerable alterations of lipoprotein metabolism and, as such, is associated with a significant increase in mortality from cardiovascular disease in this group of patients.

The dyslipidemia associated with patients with chronic renal disease is similar to the dyslipidemia of patients with insulin resistance and, as such, is characterized by an increase in plasma TG, an increase in VLDL particles and small dense LDL, and low levels of HDL cholesterol (64). DM is one of the most frequent causes of renal disease.

Further, dyslipemia contributes to the progression of renal disease. Epidemiologic studies and studies conducted in experimental animals have demonstrated that the hypercholesterolemia accelerates the progression of renal disease, and that a fat-rich diet generates an accumulation of foam cells which produce glomerular sclerosis affecting the small blood vessels of the kidneys (65, 66).

The mechanisms responsible for the hypertriglyceridemia in renal disease are, to-date, not clear although a contribution to the disease is the increase in the level of apo C-III in these patients, or the decrease in the levels of apo A-V (67). Another explanation would be the resistance to insulin that these individuals can present.

METABOLIC SYNDROME

Since about 30 years ago, there has been evidence of the combination of factors such as dysplidemia, hypertension, obesity, glucose intolerance which have been associated with an increased risk of cardiovascular disease. This led to the term metabolic syndrome (MetS) which describes individuals with an elevated risk of cardiovascular disease. The principal lipid alterations in these patients are hypertriglyceridemia and low levels of HDL cholesterol.

There are several criteria that define MetS; the principal ones are summarized in Tables 3, 4 and 5.

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NCEP/ ATPIII

Plus 3 or more:

Waist circumference (cm)	♂>102; ♀>88
TG (mg/dL)	≥150
HDLc (mg/dL)	♂<40 ; ♀<50
Blood pressure (mmHg)	≥130/85
Fasting glucose (mg/dL)	≥110

IDF

Central obesity ♂≥94; ♀≥80cm in Europeans, plus any two:

TG (mg/dL)	>150
HDLc (mg/dL)	♂<40; ♀<50
Blood pressure (mmHg)	≥130/85
Fasting glucose (mg/dL)	≥100

WHO

DM or fasting glucose or defect in glucose tolerance or IR, plus any 2:

Hip-to-waist ratio	♂>0.90; ♀>0.85
TG (mg/dL)	≥150
Or	
HDLc (mg/dL)	♂<35; ♀<39
Blood pressure (mmHg)	≥140/90
Urinary albumin excretion (µg/min)	>20
Or	
Albumin-to-creatinine ratio (mg/g)	≥30

Tables 3, 4 and 5. Summary of the criteria that, according to the three principal scientific bodies, define the metabolic syndrome.
NCEP/ ATPIII = National Cholesterol Education Program/ Adult Treatment Panel III
IDF = International Diabetes Federation
WHO = World Health Organization

As with DM2, hypertriglyceridemia in MetS is due to an increase in plasma VLDL, deficit in LPL activity, increase in CETP activity, and higher flow of fatty acids to the liver.

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OTHERS

Other causes of secondary hypertriglyceridemia have been identified, and which can be related to lifestyle, excessive caloric and carbohydrate intake, sedentary behavior, and alcohol abuse. Among other consequences, the excessive intake of alcohol inhibits oxidation of fatty acids in the liver thus increasing the amount available for the synthesis and secretion of TG-rich particles (VLDL).

Also, there are certain diseases that result in an increase in the levels of TG, such as Cushing's syndrome (68), VIH (69), hypothyroidism (70), or physiological conditions such as pregnancy (71).

Some medications can produce an increase in the levels of circulating TG, such as atypical anti-psychotic drugs (72), β-blockers (73), corticosteroids (74), estrogens (75), protease inhibitors in the treatment of HIV (76), retinoids (77), tamoxifen (78), and thiazide diuretics. In individuals with normal levels of TG, these treatments can have an irrelevant effect but, in predisposed individuals, these medications can seriously aggravate their hypertriglyceridemia.

3.2. Metabolic consequences of hypertriglyceridemia: the atherogenic dyslipidemia

The increase in the levels of circulating TG are accompanied, in humans, with other alterations in lipid metabolism that can explain its association with an increased risk of cardiovascular disease, despite the levels of LDL cholesterol being normal. These combinations of alterations are defined as atherogenic dyslipemia.

Atherogenic dyslipemia is characterized by the increase in the hepatic synthesis of large-sized VLDL ($VLDL_1$) which results, as well, in an elevation in the concentration of plasma apo B (>130 mg/dL). By the action of CETP, $VLDL_1$ generate LDL particles that are small and dense (sdLDL) and, as well, decrease the cholesterol transported by HDL. The action of hepatic lipase on VLDL, IDL and LDL generate lipoproteins that are difficult to eliminate, and are highly atherogenic remnants. In summary, atherogenic dyslipemia generates particles that under normal conditions are not abundant in the circulation, such as remnants of TRL (RLP) or sdLDL particles. HDL clearance is diminished, and is linked to metabolic alterations accompanying post-prandial hyperlipemia.

3.2.1. $VLDL_1$

Recent evidence shows that dyslipemia is principally due to the increase in intra-abdominal fat. Visceral fat increase also increases non-esterified fatty acids flow

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towards the liver (79). The liberation of fatty acids from the adipose tissue is mediated by the hormone-sensitive lipases (HSL) and adipose TG lipase (ATGL) which catalyze the hydrolysis of TG and diacylglycerides.

HSL is highly expressed in adipose tissue and in steroidogenic tissue where it controls the ratio of hydrolysis and storage of TG. In adipose tissue it is activated by hormones such as catecholamines and glucagon and is inhibited by insulin (80, 81).

ATGL which has been identified recently (82) and has lipolytic activity in adipose tissue (83). Its precise role, mechanism of action, activator and repressors are not well defined, to-date.

Under normal conditions, the fatty acids that reach the liver are, in great part, re-esterified and stored in the form of lipid droplets. Their subsequent hydrolysis is the source for the hepatic synthesis of lipoproteins and β-oxidation. The increased availability of visceral fat is the principal provider for the synthesis of the TG necessary for the formation of VLDL and the increased induction of large VLDL assembly. The concentration of large VLDL particles ($VLDL_1$) which are increased in subjects with diabetes (84) is the principal determinant of the levels of circulating TG, not only in DM2 patients but also non-diabetic individuals (85) (Figure 8).

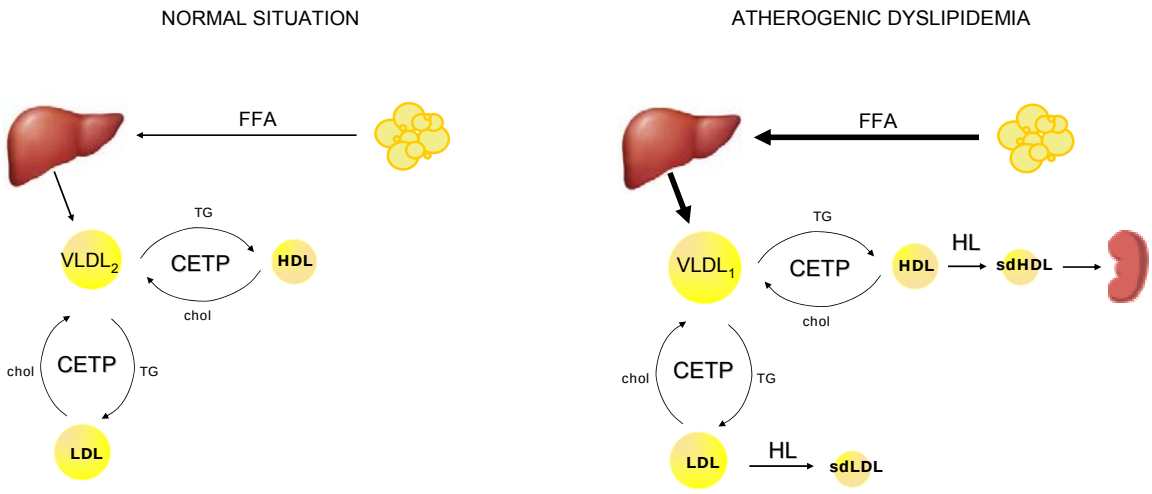

Figure. 8. Comparison between endogenous lipid metabolism in the normal physiologic situation, and under atherogenic dyslipidemia

3.2.2. Apo B levels

The increase in the transport of fatty acids to the liver, apart from stimulating the synthesis of TG and VLDL particles, also stimulates the synthesis of apo B.

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The level of apo B reflects the total number of atherogenic lipoproteins that contain apo B, although 90% of the apo B of plasma is contained in LDL (86). Recent epidemiologic studies show that apo B is a better predictor of cardiovascular disease events than cholesterol itself whether total, LDL cholesterol or non-HDL cholesterol (which is calculated as the difference between the total cholesterol and HDL cholesterol and can be considered to reflect the content of the TRL) (87). The apo B carries greater information on the interaction of the cholesterol with the arterial wall than the cholesterol itself. Further, the ratio of apo B/apo A-I has been demonstrated to be an important marker of cardiovascular disease risk (88). The system based on the apolipoproteins has greater value in predicting risk and, as has been shown in many studies, the subject does not need to provide fasting blood for its measurement.

3.2.3. Small and dense LDL (sdLDL)

In the large VLDL particles ($VLDL_1$) there is a greater proportion of apo C-III than apo C-II and, so, the LPL is not normally active. As a consequence, there is less hydrolysis of TG. The particles derived are poorly recognized by the LDL-R and the LRP which are responsible for the elimination of these particles from the circulation. This increases the time these lipoproteins continue in circulation and, due to the action of CETP (Figure 9), become enriched with cholesterol esters. The HL hydrolyzes the TG that they contain and the particles become transformed to small dense LDL (sdLDL). These sdLDL particles are highly susceptible to oxidation and are considered as highly atherogenic.

To maintain the equilibrium of oxidative status in this situation becomes especially important, and the anti-oxidants such as vitamin E play a key role. The sdLDL particles modified by oxidation are taken-up by the scavenger receptors situated on activated monocytes which become transformed into macrophages and, eventually, become foam cells. An elevated level of these particles is present in patients with metabolic syndrome.

Apart from the CETP and HL genes, association studies have demonstrated that another known genetic determinant of these particles is the APOA5 gene (89-92).

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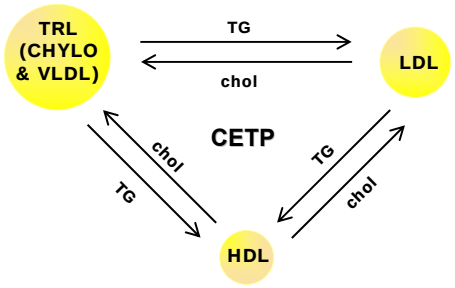

Figure 9. Schematic view of the CETP action on TRL metabolism

3.2.4. Low HDL levels

As we have seen in the section 2.3, HDL are formed from newly synthesized nascent discoidal particle that acquire cholesterol from the cell membranes via the ABC-A1 transporter. This cholesterol will become esterified via the action of LCAT, and the particle is transformed to small spherical HDL_3 and, finally, to HDL_2 which is also spherical but larger. HDL_2 can revert to HDL_3 with the participation of CETP (the enzyme that interchanges cholesterol esters and TG between HDL and the apo B containing lipoproteins) together with the SR-B1 (hepatocyte receptors that enable the selective uptake of the cholesterol esters) and the HL (enzyme that hydrolyses TG and phospholipids) (93).

Epidemiological evidence indicates that there is an inverse relationship between the levels of TG in plasma and the cholesterol transported by HDL. This relationship is especially significant in patients with MetS, DM2 and in dyslipemic patients with atherogenic dyslipidemia (94-98). As we have commented earlier, during the atherogenic dyslipidemia, due to a greater contribution of fatty acids being delivered from the visceral adipose tissue to the liver, VLDL particles rich in TG are formed and, by the action of interchange protein CETP, the plasma levels of HDLc decrease. This results in the HDL being enriched with TG and becoming lipoproteins with rapid clearance from the circulation (99) because:

- The TG that they contain can be hydrolyzed by HL forming small dense HDL particles
- Or during hydrolysis the apo A-I that they contain is lost makes these particles become unstable
- Or because under these conditions a reduction in the activity of the LPL is produce and, hence, there is a deficit of surface components derived from the hydrolysis of the TRL which serve for the formation of new HDL, and hence, less new particles are formed.

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3.2.5. Remnant lipoproteins (RLP)

The chylomicrons of intestinal origin contain apo B48. The VLDL of hepatic origin contain apo B100. Both lipoproteins are rich in TG and, due to their large size, are not associated with a significant increase in cardiovascular disease risk. But, as we had seen earlier, hypertriglyceridemia is the principal determinant of structure and size of the remnant particles; a significant and independent risk-factor for cardiovascular disease (100).

Remnant particles are formed in circulation when the chylomicrons and VLDL are converted to smaller and denser particles by the action, principally, of LPL and, to a lesser degree, by HL. These particles are poor in TG, phospholipids and apo Cs and rich in cholesterol esters and apo E. Remnant production is greatly influenced by the levels of circulating lipids, particularly TG, and of the activity of LPL, and which makes it a good candidate for the modulating action of apo A-V, as has been demonstrated in the Framingham study (101). Apo A-V, as we will see later, actively participates in the regulation of the TG levels, in part by stimulating the hydrolytic activity of LPL.

The characteristics of the remnants are what make these particles highly atherogenic. As yet it is not clear which precise physical and/or biochemical characteristics are responsible for the atherogenicity, but the following have been proposed:

- Size and number. The accumulation of lipids in the arterial wall, the critical point in the initial steps in the development of arteriosclerosis, is directly related with the quantity of circulating lipoproteins and indirectly related to the size of the particles (102).
- Apo E. The remnants are eliminated from the circulation via the receptors specific for apo E. As such, the total quantity of apo E in each particle, the individual isoform of the molecules of apo E, and the presence of other apolipoproteins in the particles can be important determinants (103).
- Oxidizability. The lipolysis of the TG-rich particles increases their susceptibility to oxidation, and the uptake of oxidized lipoproteins by macrophages is increased (104).

Remnants can be identified, separated and quantified in plasma based on their density, charge, size, specific lipid components, composition of apolipoproteins or immunospecificity of the apolipoproteins (105).

It can be difficult to differentiate these particles from their precursors because of the similar apolipoprotein composition and size. These particles can be in the postprandial circulation for long periods of time or, as well, can be converted to LDL (106).

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3.2.6. Postprandial metabolism

Postprandial status is closely linked to the metabolism of TG since the principal protagonists are the TRL (chylomicrons) derived from the ingestion of fat. As such, atherogenic dyslipimia is even more relevant during the postprandial state because of the greater number of these circulating particles contributing to increasing the levels of plasma TG.

Increasing evidence shows that an altered postprandial metabolism plays an important role in the development of atherosclerosis. Since people in developed countries are in the postprandial state for the greater part of the day, arteriosclerosis has come to be considered a postprandial phenomenon (107). The grade and duration of the postprandial triglyceridemia can be directly related with the phenomena characteristic of atherogenic dyslipidemia, such as the appearance of sdLDL, or the disappearance of HDL (108).

The evidence for mechanisms by which postprandial hypertriglyceridemia can result in atherosclerosis has been reviewed by López-Miranda et al (109). The main conclusions are:

- Increase in the concentration and residence time in circulation of the remnants of chylomicrons and VLDL that, due to their smaller size, can penetrate the arterial wall where they become toxic to the cells.
- Postprandial changes produced in the endothelium induce endothelial dysfunction (one of the initial key events in the development of arteriosclerosis). Increase in remnant particles alter the vasodilatation of the endothelium and the secretory role of the endothelium and can, for example, decrease the secretion of nitrous oxide which is a compound that regulates vascular tone and a decrease of which induces vascular relaxation.
- Postprandial TRL induce the expression of pro-inflammatory cytokines and soluble adhesion molecules (such as inter-cellular adhesion molecule-1 or the vascular cell adhesion molecule-1).
- Because of the longer residence time of the TRL, the chylomicrons and VLDL compete for the binding to LPL and this can promote the formation of the sdLDL.

The smoking habit, diet, intake of excessive alcohol, and the serum levels of creatinine have been independently associated with the quantity of postprandial lipoproteins and, as with intra-abdominal obesity, have also been associated with an increase in postprandial lipemia.

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3.2.7. Hypotriglyceridemic treatment

The optimal levels of circulating TG are 150mg/dL. The current recommendations for the treatment of hyperTG are summarized by the NCEP-ATP III (11).

The origin of hypertriglyceridemia can be due to an unhealthy lifestyle which results in excess visceral adipose tissue the resolution of which, usually, is a low-fat hypocaloric diet and physical exercise, since these measures improve insulin sensitivity. This is basic in the pathogenesis of hypertriglyceridemia because, as we have seen, insulin resistance increases the flow from the periphery of free fatty acids (FFA), and lipogenesis stimulated by insulin which leads to an increase in the hepatic secretion of TRL and which results in difficulties in clearance of the TG from the circulation. However, this treatment is difficult to achieve in practice.

In the cases of severe hypertriglyceridemia with the attendant overall elevated risk of cardiovascular disease, if there is an insufficient response to lifestyle changes, various hypolipemic agents can be used. At the clinic level, the type of treatment and the objectives differ according to the TG levels. When the levels of circulating TG are <500 mg/dL, the principal objective is to reduce the levels of LDL cholesterol and, as a secondary objective to reduce the levels of TG by lifestyle changes and/or pharmacological treatment. When the TG levels are >500 mg/dL, the principal objective of treatment with hypolipemic drugs is to prevent pancreatitis. However, as we shall see later, the criteria continue to be revised.

FIBRATES

The fibrates have a considerable effect on circulating lipids and, as the principal effect on the levels of TG and of HDLc, are especially indicated for the treatment of hyperTG associated with atherogenic dyslipemia.

The fibrates are agonists of the PPARα transcription factors that are highly expressed in tissues, and with an elevated catabolism of fatty acids.

As we have seen in section 4, the family of PPAR nuclear receptors is composed of 3 sub-types of receptors termed -α, -β/ -δ and -γ which are coded-for by different genes. Their ligands are, principally, the fatty acids and their derivatives and, in the case of PPARα, bind to exogenous ligands such as fibrates. Once activated, heterodimerization takes place with another nuclear receptor termed RXR (retinoid X receptor). Altered transcription of the genes occurs. The specific element in the DNA sequence is a repeated hexameric sequence separated by 1 or 2 nucleotides (PuGGTCA).

The fibrates promote the activation of the PPARα in the liver and the hypotriglyceridemic effect results from modifying the rate of expression of certain target

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genes which are very important in the regulation of the levels of circulating TG (revised by Fruchart et al (110)). The principal points are:

- Reduction in the secretion of VLDL by activation of genes that code for enzymes limiting the process of fatty acid oxidation, such as fatty acid transporter protein which facilitates the transport of the fatty acid across the cell membrane, and acyl-CoA synthetase which facilitates esterification and precludes their efflux from the cell.
- Increase in the lipolysis of plasma TG of the TRL. The fibrates act principally on the hepatic production of LPL, although they increase the lipolytic activity, as well. Further, they suppress the expression of the LPL inhibitor (APOC3) and, as we shall see in the following section, they regulate the expression of APOA5 that stimulates the activity of LPL *in vivo* and *in vitro*.
- Reduction in the quantity of sdLDL, mediated by the control of key genes in the formation of these particles such as LPL and CETP.
- Increase in the HDL cholesterol. Many of the key proteins in the reverse cholesterol transport are controlled by the PPARs. The activation of PPARα by the fibrates stimulates the transcription of genes such as, for example, the APOA1 (a key protein in the formation of the HDL particles) and that of APOA2 which is, as well, an element of HDL.
- In some cases of treatment with fibrates, there can be an increase in the levels of LDLc. In this case the treatment needs to be changed.

NIACIN (NICOTINIC ACID)

Niacin, or nicotinic acid, is a water soluble vitamin which, for about 50 years, has been used as a pharmacological agent for the treatment of hyperlipidemia. The effects on lipids take place, principally, following the inhibition of lipolysis in the adipocytes, inhibiting the production of cAMP (111), and reducing the levels of free fatty acids (FFA). The result is a decrease in the hepatic production and secretion of VLDL. Also, there is an inhibition of the uptake of HDL-apo A-I by the hepatocytes and, since there are more HDL apo A-I. there is an increase in the reverse transport of cholesterol, thus enabling a greater elimination of cholesterol from the vascular wall (112). Results from clinical studies suggest that the increase in the levels of HDLc promoted by niacin can be a good strategy to reduce the clinical events and to regress arteriosclerosis lesions (revised by Guyton JR.(113)).

Nicotinic acid treatment improves the lipid profile by decreasing the levels of TG up to about 50%, LDLc up to 20%, and increasing the levels of HDLc up to 25%; albeit with several secondary effects such as frequent skin rash and flushing and pruritis together

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with elevation of liver enzymes and the increase in the levels of uric acid; these latter effects are less frequent.

Drug	Total Cholesterol	LDL-C	HDL-C	TG
Fibrate	↓ 15%	↓ 0-15%	↑ 6-20%	↓ 25-50%
Niacin	↓ 25%	↓ 10-15%	↑ 15-35%	↓ 20-50%
Statin	↓ 15-60%	↓ 20-60%	↑ 3-15%	↓ 10-40%
Thiazolidinediones	↑ or neutral	↑ or neutral	↑ 10%	↓ 20-30%
Fish oil (ω3 FA)	↑ or neutral	↑ or neutral	↑ or neutral	↓ 20-50%

Table 6. Summary of the effects of hypotriglyceridemic drugs on lipid parameters

STATINS

The statins are the most powerful drugs for lowering the levels of LDLc. The mechanism is via the inhibition of 3-hydroxy-3-methylglutary-coenzyme A which is the rate limiting enzyme in the biosynthesis of cholesterol. The statins are less effective than niacin or fibrates with respect to the reduction in the levels of TG, or in increasing the levels of HDLc. In high doses, however, the recently-designed statins can reduce the TG levels but are not considered the reference treatment for the high levels of circulating TG. The mechanisms by which the statins can affect the levels of TG are not known, but it is believed that this could be an effect derived from the decrease in the levels of cholesterol in the liver, which would result in reduction of VLDL.

As commented-upon earlier, the principal target of treatment, with the exception of subjects with extremely high levels of TG, is LDLc and, secondarily, hyperTG. The characteristics hyperTG of the atherogenic dyslipidemia are accompanied by moderately elevated levels of LDLc and, as clinical evidence demonstrates, is more of a risk for cardiovascular disease that cholesterol. There is no useful clinical evidence indicating a relationship between treatment with fibrates and reduction in cardiovascular disease risk. Consequently, the FIELD study chose to treat moderate hypercholesterolemia resulting from the hyperTG.

The FIELD study (Fenofibrate Intervention and Event Lowering in Diabetes) (114) is an intervention study with fenofibrate carried-out in about 10,000 patients with DM2 followed-up for 5 years, and was designed to obtain consistent data on the capacity of fibrates to reduce the cardiovascular disease risk in patients with DM2. The principal variable of interest was the cardiovascular disease mortality. After 2 years of follow-up there were no differences detected between the study groups. The investigators

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proceeded to modify the variables-of-interest to that of fatal and non-fatal coronary events. In general, the results obtained were confusing, even in relation to the efficacy of the control of lipids.

Fortunately, over the past few years, interest in clinical studies in populations with high levels of LDLc has been replaced by populations with moderate levels of LDLc. This change results from statins having been administered without taking into account other parameters of lipid alterations and, as well, because the percentage of subjects with atherogenic dyslipidemia has greatly increased in all countries. Further, the majority of clinical studies conducted in subjects with moderately elevated levels of LDLc do not show that the statins have any selective beneficial effect in patients with atherogenic dyslipidemia (115).

THIAZOLIDINEDIONES

The first option in the treatment of DM2 is the administration of thiazolidinediones (116). Their effects on circulating TG levels are greater in subjects with non-diabetic MetS (117). The thiazolidinediones are ligands that are selective for PPARγ the nuclear receptors, and the isoform of PPARs abundantly expressed in the adipose tissue which are, as well, encountered in the pancreatic cells, in the vascular endothelium, and in macrophages. Once activated, the thiazolidinediones:

- Promote the decrease in inflammation, decreasing C-reactive protein
- Promoting the up-take of glucose, increasing the number of type 4 glucose transporter receptors
- Promote adipocyte differentiation; the uptake of fatty acids from the circulation and their storage in the adipose tissue; the decrease in intra-vascular activity of LPL in the adipose tissue; the decrease the levels of leptin (which is the hormone that is produced in adipose tissue and which regulates intake and energy expenditure) causing an increase in appetite and decreasing the metabolism; increasing the levels of adiponectin (which is the adipose tissue hormone that regulates the metabolism of the fatty acids and in inversely correlated with body fat).

Clinical studies show that treatment with thiazolidinediones decreases fasting and postprandial concentrations of glucose, as well as the levels of circulating FFA and insulin concentrations. Also, in the liver, there is a decrease in the concentrations of FFA and TG which are sources for the formation of VLDL. However, there are no published data on the molecular mechanisms explaining the effect of thiazolidinediones on lipid parameters in humans.

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OMEGA 3 FATTY ACIDS

The first evidence that demonstrated the relationship between ω-3 fatty acids and the levels of circulating TG was established by a study which proposed to evaluate why Eskimos had a lower rate of morbido-mortality from cardiovascular disease despite having a diet rich in fat. The results showed an inverse relationship between these fatty acids (present in large quantities in fish) and the levels of TG (118).

This type of polyunsaturated fatty acid is recognized by many nuclear receptors that act as transcription factors regulating the expression of multiple genes implicated in the control of lipid metabolism, such as SREBP-1c, PPARs, LXR, FXR and HNF-4α, and which can explain, via different mechanisms, its hypotriglyceridemic effects. The hypotriglyceridemic effects are due, principally, to a decrease in the hepatic secretion of TG. Hence, with less TG-rich particles in circulation, the chylomicrons compete for binding to the LPL and, hence, the ω-3 fatty acids promote the catabolism of chylomicrons. Also, they stimulate the activity of LPL (119) and this results in an overall acceleration of TRL elimination from plasma.

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4. Genetics of hypertriglyceridemia: focus on the APOA1/ C3/ A4/ A5 gene cluster

We have already seen that the apolipoproteins constitute the components of the lipoprotein particles, and are responsible for:

- Stabilizing the structure of the lipoprotein
- Guiding the lipoproteins through the circulation, enabling their recognition by proteins, receptors and enzymes necessary for their metabolism and subsequent elimination from the circulation

In 1985, the genes for the apolipoproteins A1, C3 and A4, were identified as a complex of 17 kb located on the long arm of human chromosome 11 (120). These genes regulate lipid metabolism and, as such, have been studied in depth in relation to cardiovascular disease, in humans as well as in animal models. Association studies have demonstrated the importance of this region in the control of TG metabolism, more specifically, in its transport and hydrolysis (121).

4.1. Components of APO A1/ C3/ A4 cluster

Apolipoprotein A-I is synthesized mainly in the liver and, to a lesser extent, in the small intestine. It is the principal protein component of HDL. In mice transgenic for the human gene, apo A-I has been described as having a protective effect on the development of atherosclerotic lesions while in the deficient mice there is an increase in the progression of these lesions. There are different genetic polymorphisms of this apolipoprotein (Milano, Marburg, Munster and Giesen) in which the change of an amino acid in the sequence is associated with a decrease in the levels of HDL cholesterol (122). For example, the apo A-I Milano has been described in a town in Northern Italy and the change of an Arg by a Cys in position 173 produces an anomalous protein that is associated with elevated levels of TG, low levels of HDL and normal levels of circulating cholesterol. Despite this poor lipid profile, the individuals who are carriers of this gene do not have evidence of any increased presence of arteriosclerotic lesions. Subsequently, when recombinant apo A-I Milano was administered in patients with chronic coronary syndrome, a significant regression in coronary arteriosclerosis was produced (123), suggesting that it can have a potential therapeutic use (124).

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Apolipoprotein C-III is synthesized mainly in the liver, and to a lesser extent in the small intestine. In circulation it is bound to the triglyceride-rich lipoproteins (chylomicrons and VLDL) and HDL, and its best known function is to inhibit the hydrolytic activity of LPL. The plasma concentrations of the protein correlate strongly with the levels of TG. Transgenic mice that over-express the human gene present with hypertriglyceridemia, while those that are deficient for the gene present with a decrease in the levels of TG relative to wild-type mice due, essentially, to a more rapid post-prandial clearance of TG (24).

Apolipoprotein A-IV is synthesized mainly in the small intestine. It is located in chylomicrons and HDL although, in fasting state, it is found principally in HDL. Its function it has not been well elucidated, to-date, but there are indications that it could play a role in the intestinal absorption of lipids since the intestinal synthesis and secretion of the protein increases during this process (125). Data from animal models suggest that it acts as a cofactor for lecithin: cholesterol acyl transferase (LCAT) in the reverse transport of cholesterol. In mice transgenic for the APOA4 gene and fed an atherogenic diet, the plasma levels of TG, total cholesterol, HDLc and free fatty acids are increased while non-esterified cholesterol is decreased. Also, these mice have considerably lower aortic lesions than control animals, and their HDL promote a higher efflux of cholesterol from human monocytes loaded with cholesterol. This suggests that the levels of apo A-IV can influence the metabolism of HDL, and its effects on atherogenesis (126, 127).

As such, taking account the functions of human apolipoprotein cluster, it is logical to think that the defects in the sequences of these genes can affect the synthesis and/or function of the proteins, resulting in alterations in the lipid metabolism that can promote the development of cardiovascular disease.
As such, since their discovery, there have been described allelic variants in these genes that can be used as genetic markers of cardiovascular disease risk or of intermediate markers for better and more rapid identification of risk of the disease, as has been described in several long-term studies over the past two decades (reviewed at 121). Several single nucleotide polymorphisms (SNP) described in the cluster are associated with the levels of TG, the published literature focusing on the influence of markers of APOC3. Of note among the polymorphisms of APOC3 is the G3238C change detectable using the restriction enzyme *SStI* in the 3' region of the gene, and the C-482T in the site of insulin recognition in the promoter region that is related to a

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lack of regulation of insulin can lead to over-expression of the gene and, as such, to the development of hypertriglyceridemia.

Locus		Polymorphic site/ restriction enzyme	Reference
APOA1 gene	5' flanking	XmnI (C−2500T)	Kessling AM (128) Antonarakis SE (129) Paulweber B (130)
	Intron 2	HaeIII (C317T)	Groenendijk M (131)
APOA1-C3 intergenic region		PstI	Ordovas JM (132) Antonarakis SE (129)
		T3213C	Groenendijk M (131)
		A3235C	
		T3287C	
		A5132C	
APOC3 gene	3' UTR	SstI (G3238C)	Dammerman M (133)
	Exon 3	C1100T	Xu CF (134) Ribalta J (135, 136)
	Enhancer Region	T−455C	Dammerman M (133) Surguchov AP (137) Shoulders CC (138) Groenendijk M (139) Li WW (140) Hegele RA (141)
		C−482T	
APOC3-APOA4 intergenic region		T−2854G	Waterworth DM (142) Ribalta J (143)
		PvuII	Paul-Hayase H (144) Kessling AM (145) Xu CF (146)
APOA4 gene		Gln380-His	Tenkanen H (147)
		Thr347-Ser	Boerwinkle E (148)

Table 7. Most frequent genetic variants of the human APOA1/C3/A4 cluster associated with alterations in the levels of circulating TG

Table 7 summarizes the association studies of genetic variants in the A1-C3-A4-A5 cluster region that have shown relationships with levels of circulating TG. The extensive evidence of variability in the region related to hypertriglyceridemia contrasts with the lack of functional variants that can explain these associations. In this context,

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the implication has been to identify a gene adjacent to the cluster that codes for a new apolipoprotein, the apo A-V.

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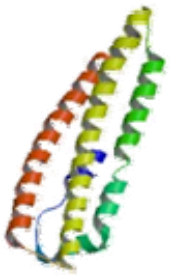
4.2. A new gene in the apolipoprotein cluster; the apolipoprotein A-V

CHROMOSOME: 11q23
GENE: APOA5
PROTEIN: apo A-V
SIZE: 366 aa, 41213 Da
FAMILY: apo A-I/ A-IV/ E



General characteristics of the gene in humans
Obtained from GeneCards, Weizmann Institute of Science (www.genecards.org).

4.2.1. APOA5 gene identification

In 2001, Pennacchio et al (149) searched for potentially functional sequences around the APOA1-C3-A4 cluster that were evolutionally conserved. In comparing human and mouse sequences they identified a new gene about 30 kb from the cluster.

They demonstrated that the genomic sequence in the mouse consisted of 3 exons and coded for 368 amino acids, the sequence of which had considerable homology to that of mouse apo A-IV (24% identity, 49% similarity). The equivalent human sequence coded for 366 amino acids and had high degree of homology to the mouse sequence (27% identity 48% similarity) and, so, was termed apo A-V. They predicted a protein structure with various amphipathic helical domains and an n-terminal signal peptide, characteristics of the proteins that bind to lipids.

The analysis of the gene expression profile in different mouse (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testicles) and human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas) showed that it was expressed mainly in the liver in both species.

To examine its function, transgenic mice and mice deficient in the human gene were generated. They analyzed the levels of circulating cholesterol and triglycerides in both animal models and observed that the transgenic mice had a third of the level of TG, and the KO mice, or the mice deficient for the gene, had four-fold more TG that the normal control mice.

The distribution of the plasma lipoproteins in the mouse models showed that the apo A-V of the mouse is distributed in VLDL and HDL and that the VLDL particles were elevated in the KO mice and decreased in the transgenic mice, relative to the normal

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controls. The size of the particles were very similar in all the animals, which implies that there were greater numbers of VLDL particles in the KO mice and lower numbers in the transgenic mice.

To extend this interesting work, they identified 4 genetic variants in humans which they assigned as SNP 1-4 and which they used in association studies (Table 8). In approximately 500 Caucasian normolipidemic subjects they observed a significant association between the minor alleles of the SNPs 1-3 and higher levels of plasma TG (increased by 20-30%) and the TG of VLDL. The 3 SNPs were in linkage disequilibrium, but not so with the SNP4 nor the marker of APOC3 *SstI*, the most consistent association to TG in this region. Hence, neither SNP4 nor SstI were responsible for the effect on TG of the APOA5 haplotype in this population. The results obtained with the SNP3 were replicated in a second population stratified by levels of TG. They observed a higher frequency of the heterozygous genotype in the group of subjects with higher levels of TG. As such, they proposed using the polymorphisms of APOA5 as prognostic indicators of susceptibility to hypertriglyceridemia, and the modulation of the apo A-V as a possible strategy to reduce the risk of cardiovascular disease associated with TG.

	Polymorphic site	**Minor allele frequency (%)**	**SNP ID**
SNP1	c.1259T>C	9.1	rs2266788
SNP2	IVS3+476G>A	8.4	rs2072560
SNP3	-1131T>C	9.2	rs662799
SNP4	-12238T>C	36.3	---

Table 8. Summary of the nucleotides, minor allele and identifier rs of the 4 SNPs studied. Adapted from Pennacchio et al (149)

Almost simultaneously to the publication by Pennacchio et al, there was another publication describing the discovery of the apo A-V (150). In this case the objective was to explain the molecular mechanisms underlying liver regeneration in hepatectomized rats. For this purpose the genes that were over-expressed 6 hours after the resection of 70% of the liver were selected. The choice of 6 hours was because this represents the mean time (between 4 and 8 hours) post-hepatectomy that the genes of the cell cycle are activated. Of all the genes that they observed to be over-expressed relative to the control levels, there were 3 unrecognized genes which were of interest because the most over-expressed was RAP-3 (regeneration-associated protein). They also studied the profile of expression of the gene in various mouse (skeletal muscle, spleen, liver, kidney, heart, brain and lung) and human (brain, heart, skeletal muscle, colon, spleen,

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thymus, heart, colon, kidney, small intestine, placenta, lung and leukocytes) tissues and showed that RAP-3 was expressed principally in the liver. They observed that the sequence of RAP-3 was very closely homologous to the cDNA of mouse and rat, and that the human cDNA was identical to certain parts of the 11q23 region where the apolipoprotein A1-C3-A4 cluster is located.

The protein sequence was observed to be very homologous between species (73%) and had certain homology with apo A-IV and apo A-I. There were α-helix domains identified as well as a signal peptide that could be liberated. This implied that the protein could be secreted into the circulation. They raised monoclonal antibodies and detected a concentration of the protein in plasma of the normal mouse of 1μg/ml bound, mainly, to large HDL. As such, they hypothesized that at the moment in which RAP-3 is most over-expressed (at 6h post-hepatectomy) there would be, as well, the highest levels of HDL. However, they observed the converse i.e. that the level of HDL protein was decreased and the level of HDL cholesterol remained constant. As such, they postulated that it could have a specific function during regeneration, possibly as an antagonist to the hepatic uptake of lipids

Van der Vliet et al in 2002 (151) published a study comparing the lipid profile in mice with normal levels of expression of APOA5, and mice that had 20-fold expression of the gene using adenovirus vectors. They showed that those that over-expressed the gene had, apart from the effect already described on the levels of TG (decrease of 70% due to the lower content in the VLDL), a decrease of 40% in the levels of cholesterol (mainly in the HDL fraction).

In summary, a new gene, APOA5 has been identified in the A1-C3-A4 cluster that appears to have a key function in the regulation of the circulating levels of lipids. It is important to highlight that although the initial studies in animal models clearly showed its importance in the metabolism of TG, the overall physiological function and mechanism of action are still unknown.

4.2.2. Implication of apo A-V in TG metabolism: *in vitro* studies and animal models

In 2003 there were two studies which used computer modeling of structural data to speculate on the function of apo A-V. The studies described that, due to its composition, the apo A-V is a protein that is highly insoluble and that it is interchangeable, as with other proteins (such as, for example, apolipoproteins A-I, C-III

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and A-IV), it can form lipid molecules together in combination with phospholipids and, as such, maintain its solubility in plasma by interacting with the lipoproteins. It was described as being bound to HDL and its capacity to activate the enzyme LCAT and to stimulate cholesterol efflux from the cells via the transporter ABC-A1 was investigated. The conclusions were that it was not implicated in any of these processes (152). Weinberg et al (153), documented its highly hydrophobic nature and suggested that, in plasma, it needed to be bound to lipoproteins. Taking into account that the concentrations in plasma is very low around 0.1% of that of apo A-I (150), the authors proposed that the negative correlation between the expression of APOA5 and the levels of TG detected in animal models was mediated intra-cellular rather than intra-vascular processes. This makes biological sense if we take into account that the apo A-V participates in the process of liver regeneration, and that certain homology has been observed between the c-terminal end of the sequence of the APOA5 gene and that of mouse MTP, and which suggests a possible participation in the process of synthesis and secretion of TGL particles.

Despite the lack of functional studies, the predictions based on the sequence analyses postulate that apo A-V delays the second stage of the assembly of VLDL particles making it difficult for the particle to expand, or restricting the nascent secretion pathway (154).

Two subsequent studies described one of the mechanisms by which apo A-V affects the levels of circulating TG. Schaap et al (155) used adenovirus transfection to increase the levels of APOA5 expression in mice. They observed an increase in the clearance of circulating TRL which was dependent on the hydrolytic action of LPL and the presence of apo C-II (principal activator of LPL). They concluded that recombinant apo A-V stimulated the hydrolytic activity of LPL *in vitro*. Also, they analyzed the role of apo A-V on the synthesis of particles and observed that over-expressing the gene gives rise to a decrease in the size and the content of the VLDL, but without affecting their rate of production. This accord with the postulate by Weinberg et al (153) which proposes that apo A-V restricts the process of lipidation or secretion of the TRL in the liver.

Fruchart-Najib et al (156) enriched human VLDL particles with recombinant apo A-V and also observed that the hydrolytic activity of LPL was stimulated by the apoprotein. They generated mice transgenic for the human APOA5 gene and observed that, in the

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fasting state, the protein is preferentially associated with the HDL particles and was transferred to VLDL in the post-prandial state.

Two further studies explored the process of activation of LPL by the APOA5 gene, by using animal models of mice that over-expressed the human gene and in knockout (KO) mice. They concluded that apo A-V accelerated the catabolism of chylomicrons and VLDL without affecting their production and that *in vitro* and *in vivo* the LPL can be bound to a proteoglycan that is activated by the apo A-V (157). However, an *in vitro* study by Lookene et al concluded that apo A-V is not capable of directly activating LPL, but this finding was probably due to the absence of proteoglycans in the design of their experiment (158).

Similarly, 2 mutations were identified in the gene which resulted in the formation of a truncated protein in the positions 148 and 139 (Q148X and Q139X). These were associated with alterations in the catabolism of the TRL due to an inability to activate LPL leading to severe hypertriglyceridemia (159, 160). Following a similar line of work, Sun et al demonstrated that the domain of apo A-V that is necessary for the interaction with LPL is localized between the amino acid residues 192 and 238 (161).

The possible mechanisms of Apo A-V function are schematically presented in Figure 10.

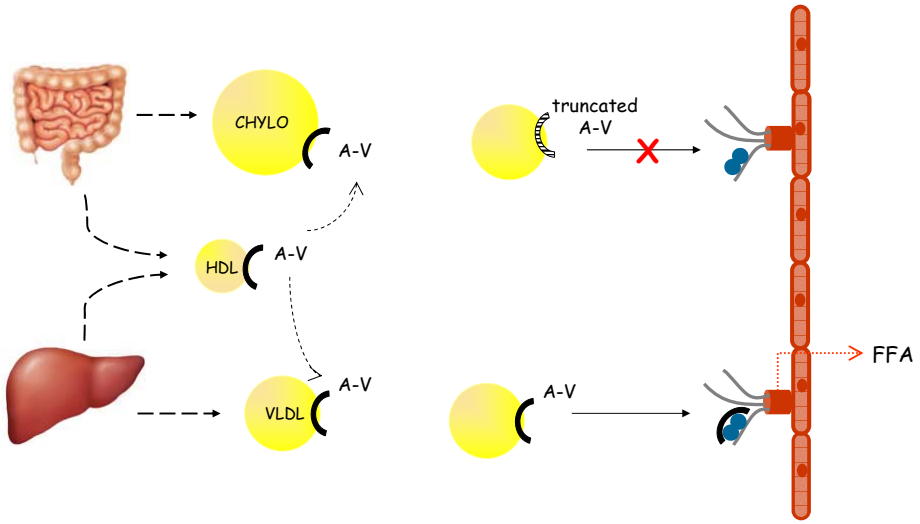

Figure 10. Possible mechanisms of action of apo A-V. The apo A-V is transported in the TRL (chylomicrons and VLDL) and in HDL (162). Apo A-V is synthesized in the liver and is secreted together with VLDL, such that the protein present in chylomicrons need to derive from synthesis in the intestine together with the particles or that post-prandially are transferred from the HDL to the chylomicrons of intestinal origin (156). Once in circulation, it facilitates the binding of the TRL to the proteoglycans making the particle accessible to the hydrolytic enzyme LPL. It is not clear if the apo A-V stabilizes the dimeric conformation of the LPL or if it is bound to the allosteric site of the LPL to stimulate its activity and to promote the formation of fatty acids from the TG contained in the particles. In any case, the presence of mutations such as Q145X or Q139X causes the protein to lose its function and results in an accumulation of the triglyceride-rich particles in the circulation.

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A further hypothesis is that apo A-V can be the key protein in the interaction between chylomicrons and GPIHBP1, which is known to play an important role in the hydrolytic process of chylomicrons (163) since it is localized in the lumen of the capillaries and can bind with LPL, and also since mice deficient in this protein develop chylomicronemia (164).
Recently, another mechanism of control of circulating levels of TG has been identified. Grosskopf et al (165) showed that a deficit of apo A-V attenuates the elimination of remnants of the TRL, since they have a lower affinity for the LDL receptor. Nilsson et al (166) characterized this interaction between apo A-V and the receptors of familial LDL-R and observed that binding is with the LDL receptor related protein (LRP) and the type-1 receptor mosaic (SorLA).

In summary, *in vitro* studies as well as studies in animal models have demonstrated that apo A-V stimulates TG hydrolysis via LPL; the process requiring the presence of apo C-II. Apo A-V is bound to the proteoglycan heparin sulfate and, further, facilitates the elimination of the remnants of the TRL.

4.2.3. Apo A-V and inflammation

Apart from its role in regulating the levels of plasma TG, several research groups have explored the possible involvement of the gene in other processes linked to hypertriglyceridemia, such as inflammation. Apo A-V, together with apo A-IV, are proteins that are present in high concentrations in HDL of mice that present with acute phase reaction related with the process of infection and inflammation (167). During the acute phase response there is an increase in hypertriglyceridemia produced due to an increase in hepatic synthesis of TG and of the secretion of VLDL. The changes in many of the proteins associated with HDL during the acute phase response serve as a defense, and are not related with lipid metabolism.
Studies in mice as well as in humans have corroborated these findings i.e. that during the acute phase response, plasma apo A-V is not inversely correlated with the levels of TG, and that the hepatic expression of APOA5 is regulated principally by changes in the stability of mRNA (168).
Apo A-V can, as such, have other functions not so intimately linked to lipid metabolism, but may be equally important in the development of cardiovascular disease.

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4.2.4. APOA5 gene variants: impact on TG metabolism

Since the discovery of the APOA5 gene, there have been many association studies with polymorphisms of the gene. There have been 5 haplotypes defined by 7 polymorphisms described (Table 9). The haplotypes APOA5*2 and APOA5*3 are the most interesting because of their association with higher concentrations of TG. The majority of studies have focused on the 2 polymorphisms -1131T>C (corresponding to the SNP 3 of the original study by Pennacchio) and the c.56C>G (also known as S19W) (169-177).

Haplotype	-1131T>C (SNP3)	c.-3A>G (Kozak)	IVS3+476G>A (SNP2)	c.1259T>C (SNP1)	c.56C>G (S19W)	1764C>T	c.457G>A (V153M)	Freq.
APOA5*1	T	A	G	T	C	C	G	69%
APOA5*2	Rare	Rare	Rare	Rare	C	C	G	4%
APOA5*3	T	A	G	T	Rare	C	G	4%
APOA5*4	T	A	G	T	C	Rare	G	---
APOA5*5	T	A	G	T	C	C	Rare	---

Table 9. Summary of gene variants determining the commonest APOA5 haplotypes. Frequency determined in Caucasian populations. Adapted from (176).

Since the APOA5 gene is located very near the APOA1-C3-A4 gene cluster on chromosome 11q23 (figure 11), there have been many studies suggesting that the effect of apo A-V on the TG levels may reflect a linkage disequilibrium with functional variants of other genes in the cluster. The Sst-I variant has previously been shown to be associated with elevated levels of TG and is in strong linkage disequilibrium with -455T>C and -482 C>T, both located in the response-to-insulin site in the promoter of APOC3 (133). While other (178, 179) studies have demonstrated that the S19W variant of APOA5 is independent of the effect of APOC3 on the effect on TG levels, there have been studies showing that -1131T>C is in strong linkage disequilibrium with the -482C>T variant in APOC3. Hence, it became necessary to conduct studies on functionality of the principal variants of the APOA5 gene.

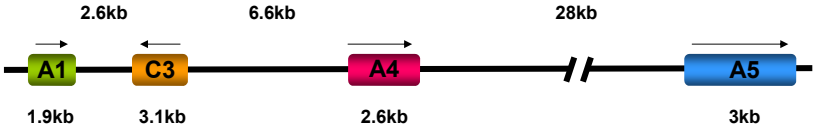

Figure 11. Map of the gene cluster. Arrows indicate the direction of transcription, and numbers indicate gene size or distance.

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Talmud et al (180), in 2005, published an extensive study describing the studies of functionality for most frequent variants of APOA5. In the case of the S19W polymorphism in the signal peptide region they demonstrated that it was necessary for the secretion of the protein and, as such, was functional. But in the case of the -1131T>C variant, using studies of luciferase activity, they did not find any significant differences in the activity between the normal and the mutated vectors (-1131T and -1131C, respectively), although both showed almost double the luciferase activity compared to the control vector. Further, since -1131T>C variant is in complete linkage disequilibrium with the -3A>G mutation i.e. the Kozak region of the promoter, they designed constructs that covered the two variants. They did not detect significant differences between the normal construct and that which contained the changes although, again, the constructs had higher luciferase activity than the control constructs. They came to the conclusion that the -1131T>C variant did not modify the transcriptional activity of the promoter. However, a few years later while studying the combination of three variants that describe the APOA5*2 haplotype and which are in complete linkage disequilibrium (-1131T>C, -3A>G, and c.1259T>C), they observed that these polymorphisms act cooperatively and that together they show significant differences in luciferase activity (181).
In conclusion, identification of the -3A>G as being functional implied that, probably, the associations described for the SNP -1131T>C reflect the effect of -3A>G.

The polymorphisms in APOA5 have been analyzed in several different population studies and have corroborated the influence of the gene on lipid metabolism, especially that of TG (Table 10).

Population	Sample size	Reference	Associated with
Healthy normolipidemic	n= 501	Pennacchio LA (149)	TG, VLDL mass
	n= 419	Pennacchio LA (177)	TG, VLDL mass, apo B
	n= 2808	Talmud PJ (178)	TG, HDLc
	n= 89	Ribalta J (182)	--
	n= 500	Li GP (183)	TG, TC, HDL-C
	n= 2391	Lai CQ (101)	TC, TG, HDL-C, LDL-C, apo B, RLP-C, RLP-TG, LDL diameter, apo A-I, VLDL
	n= 196	Ishihara M (184)	TG, apo A-V
	n= 299	Sundl I (185)	VLDL-C, IDL-C, TG, VLDL-TG, vit E, apo B/apo A-I
Schoolchildren	n= 552	Endo K (186)	TG, HDLc
General population	n= 893	Nabika T (187)	TG

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	n= 2660	Pennacchio LA (177)	TG
	n= 408	Ribalta J (182)	TG
	n= 3971	Lai CQ (188)	TG, LDL-C, HDL-C
	n= 558	Austin MA (89)	TG, LDL size, apo B
	n= 2559	Hubacek JA (189)	TG
		Hubacek JA (190)	HDL-C
	n= 285	Hubacek JA (191)	TG
	n= 3831	Klos KL (192)	TG
	n= 1119	Hubacek JA (193)	--
	n= 3020	Hodoglugil U (194)	TG
	n= 2148	Lai CQ (195)	TG, RLP, VLDL size, LDL size
	n= 794	Chandak GR (196)	TG, TC
	n= 117	Hubacek JA (197)	TC
	n= 2273	Elosua R (174)	IMT, TC, TG, HDL-C, obesity
	n= 2490	Talmud PJ (198)	TG, apo A-V
	n= 1183	Hallman DM (173)	TG
	n= 3794	Yamada Y (199)	TG, HDL-C
	n= 2280	Corella D (200)	obesity
	n= 3124	Grallert H (170)	TG, TC, HDL-C, LDL-C
	n= 2500	Hubacek JA (201)	TG
	n= 477	Liao YC (202)	TG, HDL-C
Oral fat load	n= 88	Masana L (203)	Fasting TG, AUC-TG
	n= 158	Jang Y (92)	TG, dense LDL, CRP, isoprostanes, DNA damage, PPL CHYLO-TG, PPL VLDLTG
	n= 51	Moreno R (204)	PPL TG, PPL TRL-TG, PPL TRL-C, PPL TRL-RP
	n= 88	Moreno R (205)	PPL TG, PPL TRL-TG, PPL TRL-C, apo B100
	n= 259	Olano-Martin E (206)	Fasting TG, AUC-TG, IAUC-TG, HDL-C
Oral fat and glucose load	n= 774	Martin S (207)	TG, waist to hip ratio, SBP, AUC-insulin, peak insulin.
Dyslipidemic	n= 459	Pennacchio LA (149)	TG
	n= 103	Ribalta J (182)	TG, VLDL-TG, IDL-TG, VLDL-C, VLDL, apo B
	n= 627	Aouizerat BE (208)	TG, VLDL-TG, VLDL-C, LDL-TG, HDL-TG, HDL-C
	n= 83	Vrablik M (209)	TG
	n= 915	Evans D (210)	TG, HDL-C
	n= 290	Kao JT (211)	TG
	n= 167	Baum L (212)	TG, HDL-C

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	n= 221	Bertolini S (213)	TG
	n= 127	Esteve E (90)	sdLDL
	n= 372	Talmud PJ (214)	TG, VLDL mass, IDL-TG, IDL-C, IDL-free C, IDL-phospholipids
	n= 298	Mar R (91)	TG, LDL size
	n= 202	Jiang YD (215)	TG
	n= 308	Chaaba R (216)	TG
	n= 285	Yan SK (217)	TG, HDL-C, apo C-II, apo C-III, apo E
	n= 606	Aberle J (218)	Weight
	n= 72	Evans D (219)	--
	n= 215	Dallinga GM (220)	Apo A-V
	n= 71	Zhai G (221)	TG, LDL-C
	n= 100	Choi JR (171)	TG
	n= 201	Maasz A (222)	TG
	n= 157	van der Vleuten GM (172)	TC, TG, apo B, HDL-C, sdLDL, RLP-C
	n= 902	Qi L (223)	TG, HDL-C
	n= 791	Lai CQ (224)	TG, HDL-C
	n= 119	Henneman P (225)	Apo A-V
	n= 279	Niculescu LS (226)	BMI, TG, HDL-C, glucose
	n= 1708	Dorfmeister B (227)	TG
	n= 1017	Yamada Y (228)	TG, HDL-C
	n= 169	Girona J (229)	Vit E, TG, apo A-I, VLDL-TG, HDL-TG
	n= 213	Kisfali P (230)	TG
	n= 308	Chien KL (231)	TG
Pregnant women	n= 483	Ward KJ (232)	TG, total cholesterol, maternal height, fetal length
CAD	n= 308	Szalai C (233)	TG
	n= 537	Lee KW (234)	TG, FER-HDL, HDL-C
	n= 312	Bi N (235)	TG
	n= 483	Liu H (236)	TG, HDL-C
	n= 1703	Ruiz-Narvaez EA (175)	TG, HDL-C
	n= 442	Dallongeville J (237)	TG
	n= 211	Hsu LA (238)	TG, HDL-C
	n= 232	Tang Y (239)	TG
	n= 1034	Vaessen SF (240)	TG, apo A-V
	n= 669	Martinelli N (179)	TG, HDL-C, apo C-III
HIV	n= 229	Guardiola M (241)	BMI, lipodystrophy, TC, TG
	n= 438	Arnedo M (242)	TG, HDL-C
Elderly	n= 371	Chen ES (243)	Obesity

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	n= 1419	Yamada Y (199)	TG, HDL-C
Nephropathy	n= 367	Baum L (244)	TG, HDL-C
Mental diseases	n= 284	Papassotiropoulus A (245)	TC
	n= 302	Havasi V (246)	TG
	n= 106	Barbosa FA (247)	--
	n= 189	Smith RC (248)	TC
	n= 403	Maasz A (249)	TG

Table 10. Summary of the association studies conducted with the APOA5 gene variants

Apart from the consistent associations with the TG levels, several studies have shown that the variants in APOA5 are associated with other parameters of interest in the study of cardiovascular disease and the associated risk factors such as the levels of total and HDL cholesterol, levels of cholesterol and TG in circulating remnant particles, levels of C-reactive protein, of isoprostances, size and density of LDL, insulin sensitivity and β-cell function,... (reviewed in (250)).

4.2.5. APOA5 gene variants: impact on apo A-V protein levels

Since 2005 there began to be published studies determining the concentrations of circulating apo A-V. O'Brien et al (162) described that apo A-V concentrations in humans were very low (Table 11) and that, as has been indicated, they are observed bound principally to the TG-rich lipoproteins (chylomicrons and VLDL) and HDL.

Taking into account the inverse relationship between the gene and circulating TG levels described in several studies in animal models (149-151, 251, 156, 252, 165), and taking into account as well the results of functional studies, we would expect to observe an inverse relationship between the circulating concentrations of apo A-V and TG. However, the majority of studies have shown no correlations, or even positive correlations (220, 253, 254); findings that suggest that the secreted protein is not the route by which the apo A-V regulates the concentrations of TG and, perhaps, that the relevant function of apo A-V in intra-cellular.

Apolipoprotein	Plasma concentration
Apo A-I	100-150 mg/dL
Apo C-III	8-15 mg/dL
Apo A-IV	30-50 mg/dL
Apo A-V	15-25 µg/dL

Table 11. Plasma concentrations of apolipoproteins that form the cluster

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A study conducted in 196 healthy individuals and in 106 diabetic Japanese patients showed that the individuals who were carriers of the wild type variant -1131T>C had higher concentrations of protein than the carriers of the less frequent -1131C allele (184). In another study, the carriers of the -1131C and c.56G alleles were associated with lower levels of circulating apo A-V and to a higher risk of cardiovascular disease; the affect was independent of the TG levels and of linkage disequilibrium with APOC3 variants (240). A third study that analyzed the two most commonly observed polymorphisms noted that only the carriers of the S19W variant had higher levels of A-V in plasma. The authors concluded that the effects described for the -1131T>C marker are due to the linkage disequilibrium between the APOC3 gene markers (198).

4.2.6. APOA5 gene regulation: implication of nuclear receptors

The expression of the apo A-V gene is under the control of nuclear receptors; transcription factors that respond to hormones and certain molecules such as drugs, or vitamins that act as ligands. The binding with its ligand promotes certain conformational changes, and activates a metabolic cascade to maintain homeostasis of lipids by controlling transcription, by binding directly to DNA of certain genes implicated in metabolism, as well as the storage, transport and elimination of lipids.
The principal receptors implicated in lipid metabolism are PPAR, FXR, LXR, SREBP and ROR. The majority of these regulatory elements are strongly related to the regulation of levels of circulating TG. Over recent years, several authors have tested whether the effect of these nuclear receptors on the TG are mediated via APOA5.

PPAR. PPARs are a sub-family of transcription factors that regulate the expression of certain genes involved in TG levels regulation (255, 256) via response to ligand binding (fatty acids are natural ligands for PPARs) and, following the binding, leading to a trans-activation or a trans-repression of target genes. Thus, the modulation of gene transcription is due to the binding of a heterodimer to a specific DNA sequence; the peroxisome proliferator responsive element. This consensus sequence consists of a direct repeat of a hexamer AGGTCA separated by a nucleotide (DR1) with a 5' extension (AACT). This system sequence recognition is, in considerable part, common for the other types of nuclear receptors.
The sub-family is composed of three different types (-α, -β/-δ and -γ), and each one is encoded separately (chromosomes 22q13, 6p21 and 3p25, respectively) and have different tissue distribution patterns (PPAR-α and -γ are predominantly expressed in liver and adipose tissue, while PPAR-δ is ubiquitously present throughout the

organism, but to a lesser extent in the liver). They have different regulatory functions in the energy metabolism (Figure 12).

These nuclear factors are of clinical interest since they are molecular targets for fibrates (main pharmacological agents to regulate TG levels) and for thiazolidinediones (used in hypoglycemic therapies) (116).

In 2003, the activity of the promoter of the APOA5 was described as being stimulated by the binding of the nuclear transcription factor PPARα (257). Further, it was demonstrated in *cynomologus* monkeys that the administration of a PPARα agonist over 14 days increased the levels of A-V protein in plasma and, as would be expected, decreased the circulating levels of TG (258). An association study demonstrated that the carriers of the S19W variant had a better treatment response with fenofibrate in terms of lipid parameters than did individuals with the normal genotype (224).

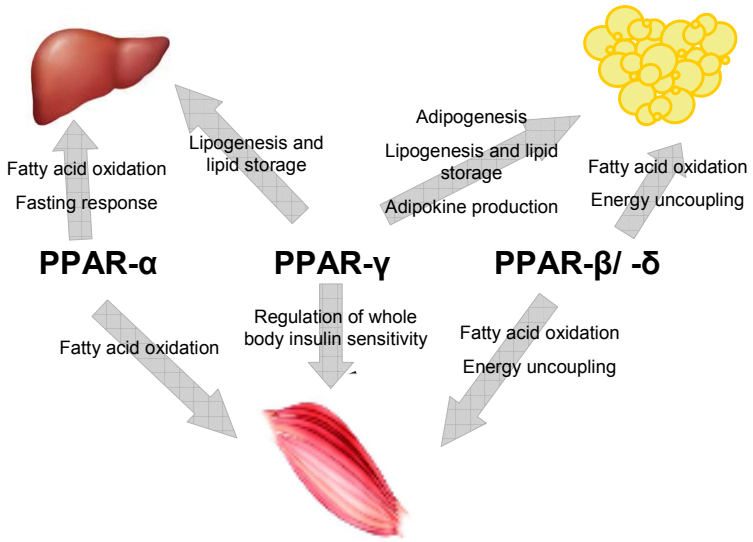

Figure 12. The PPAR isoforms regulate lipid and glucose metabolism through their action on different tissues

FXR. FXR is identified as a nuclear receptor activated by bile acids and expressed, above all, in the enterohepatic system. It protects the organism from the consequences of elevated concentrations of bile acids. It is related to the metabolism of cholesterol since it inhibits the synthesis of bile salts (Figure 13). FXR has been described as a key regulator in the control of plasma TG levels (259) and, when activated by a specific ligand, promotes a 50% decrease in plasma TG levels in rodents (260). In 2003, Prieur et al demonstrated that FXR was a regulator of TG levels which, in large part, was mediated by the control of APOA5 expression (261).

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LXR. LXR is expressed in the liver, but also in other tissues involved in lipid metabolism such as adipose tissue, kidney, intestine, macrophages. It is a nuclear receptor that binds cholesterol derivatives and, in response to an increased concentration of cellular oxysterols, modulates the expression of genes involved in cholesterol metabolism (Figure 13). Further, it regulates a number of genes involved in fatty acid and TG metabolism by up-regulating another nuclear receptor, the SREBP-1c. In 2004 a study was published demonstrating that one of these target genes is APOA5, and that LXR via SREBP-1c negatively regulates the levels of expression of the gene and of the protein (252).

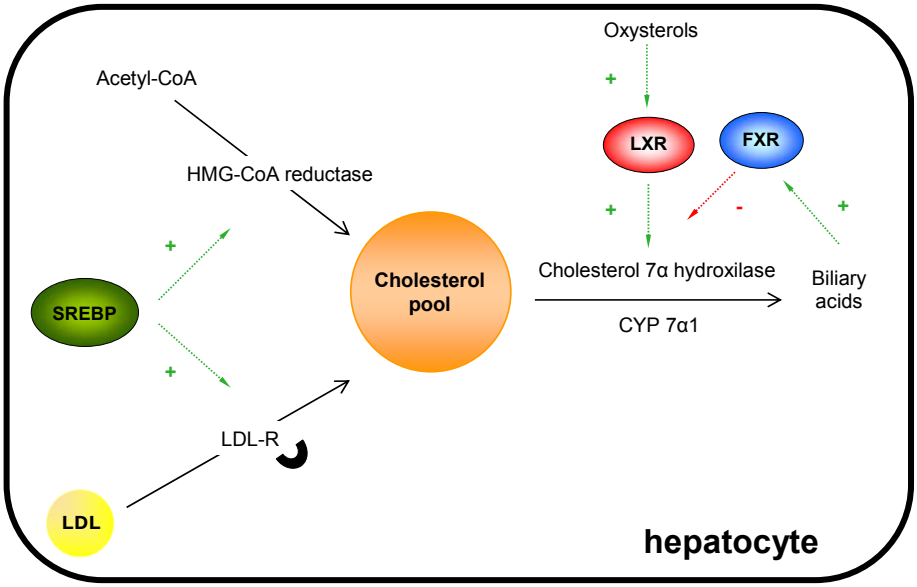

Figure 13. The nuclear receptors only act in coordination and, for example, represent the transcription factors implicated in homeostatic control of cholesterol. SREBP stimulates the formation of LDL-R and the key process in the synthesis of cholesterol (HMG-CoA reductase) and, in response to the presence of cholesterol derivatives, the LXR stimulates the formation of bile acids; a process that is also controlled by FXR.

SREBP. The SREBP is composed of three isoforms: SREBP-1a and SREBP-1c which are coded by the same gene on human chromosome 17 and, due to alternative promoters, form transcripts with 6 different exons; SREBP-2 which is located on chromosome 22 (262). These transcription factors are known principally for their roles in the control of cholesterol homeostasis. *In vivo* studies using animal models have shown that SREBP-1c is preferentially involved in the control of metabolism of bile acids and TG, and is the only member of the family that is activated by LXR and insulin (263). The activation of SREBP-1c is also modulated by food intake; a characteristic that may relate apo A-V with nutritional status.

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Insulin. Insulin is a molecule implicated in the response to nutritional status and, as such, is an important factor in the regulation of metabolism of carbohydrates and lipids in the liver, adipose tissue and muscle. In relation to the lipids, it is implicated in the oxidation of fatty acids in the liver, in lipogenesis, in the synthesis of proteins, and in the export of fatty acids in the lipoproteins from the liver to the extra-hepatic organs, among the functions. Also, the possible regulatory role in APOA5 expression by insulin, has been described (264). The authors suggested that the metabolic mechanism occurs as a function of upstream stimulatory factors (USF). Insulin activates phosphatidylinositol 3-Kinase (PI3K) which phosphorylates the USF that affects the DNA sequence of the APOA5 gene causing a decrease in the transcription levels. Also, insulin infusion reduces the levels of apo A-V protein.

ROR. The retinoic acid-related orphan receptor (RORα) (265) was considered not to have any exogenous activator, although recently it has been described that cholesterol or its derivatives are its natural ligands. Once activated, RORα is involved in many aspects such as smooth muscle cell differentiation, inflammation, lipid metabolism and the regulation the levels of plasma cholesterol. RORα-deficient animal models have a high susceptibility to arteriosclerosis. Although its physiologic function has not been clearly established evidence suggests that it modulates the expression of genes such as APOA1 (266) and APOC3 (267). It has been shown to stimulate APOA5 promoter transcriptional activity (265, 268) and the modulation of the expression of other components of the apolipoprotein cluster needs to be investigated.

TH. Hypothyroidism is a disease associated with elevated levels of circulating TG which, in turn, is associated with low activity of LPL. Hence, thyroid hormone status has been widely investigated in relation to the metabolism of the triglyceride-rich particles. The relationship between the hormone and TG leads one to believe that the thyroid hormone can regulate the expression of APOA5. For example, treatment of human primary hepatocytes with T3 increases the levels of mRNA and, as well, of apo A-V protein and results from the binding of a response element in the APOA5 promoter (269).

HNF-4α. The fatty acids, as well, are ligands for HNF-4α which is expressed principally in the liver, but as well in the kidney, intestine and the pancreas. It plays a key role in the differentiation of the hepatocytes during development in the liver and, as well, in the regulation of the expression of genes implicated in the metabolism of

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glucose and of lipids, including the APOA1-C3-A4 cluster, APOB, MTP, among others. The inhibition of HNF-4α in human hepatocytes results in the down-regulation of APOA5 and indicates that this nuclear receptor directly regulates human APOA5 promoter activity (270).

Apart from the transcription factors, there have been studies assessing the role of enhancer of APOC3. The APOC3 enhancer is a common regulator element of the human apolipoprotein A-I/ C-III/ A-IV/A-V cluster which regulates the specific tissue expression of APOA1, C3 and A4 genes (271). Studies conducted in mice transgenic for the whole cluster region show that the APOA5 is the only one of the cluster that is not co-regulated by the enhancer of APOC3 (272) and it is postulated that this can explain the big differences in the levels of circulating protein between the components of the cluster. A conclusion is that the control mechanism of APOA5 expression is more complicated than originally thought.

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Hypothesis & Objectives

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Hypothesis

Since apolipoprotein A-V is a key modulator of plasma triglyceride levels it should also have an effect on different aspects highly linked to TG metabolism, such as fat-soluble vitamins or the effect of certain TG levels-modifying drugs, and its effect should be related with synthesis and secretion of TG-rich lipoproteins.

Objectives

The proposed objectives to test this hypothesis were:

1. Use variability at the APOA5 locus to study its influence on lipid, lipoproteins, fat-soluble vitamins and oxidative status; and the interaction with drugs in different populations.
   1.1. In healthy men (complete lipid, lipoprotein and fat-soluble vitamins profile)
   1.2. In diabetic patients (complete lipid, lipoprotein and oxidative status profile)
   1.3. In HIV patients (treated with protease inhibitors)

2. Study the APOA5 gene function
   2.1. Study if APOA5 gene is modulated by diet (in KO-APOE mice)
   2.2. Study whether it is expressed in the intestine (in TC7/ Caco2 cells)

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Results

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Results

This section summarizes the 5 studies carried out in the course of this thesis development, and which are written in article format. Two of these studies are presented as the articles used for assessment of the thesis (Clinical Chemistry 2006; Clinical Chemistry and Laboratory Medicine 2008), and the other studies are presented as annexes (Journal of Lipid Research 2007; Clínica e Investigación en Arteriosclerosis 2007; Biochimica et Biophysica Acta, Submitted 2008). The data are grouped in two sections; association studies and functional studies.

ASSOCIATION STUDIES:

Data acquisition for the thesis began by evaluating the influence of the APOA5 gene on TG metabolism described in the literature extant at that time. For this purpose we used association studies of the -1131T>C gene variant in our population samples. This variant has been the most commonly used in studies conducted in different populations which were well-characterized with respect to different aspects of lipid metabolism. The results of our findings were:

1) Increased concentrations of circulating vitamin E in carriers of the apolipoprotein A5 gene -1131T>C variant and associations with plasma lipids and lipid peroxidation.
   Isabella Sundl, Montse Guardiola, et al.
   Journal of Lipid Research 2007;48:2506-2513.
2) The apolipoprotein A5 gene -1131T→C polymorphism affects vitamin E plasma concentrations in type 2 diabetic patients.
   Josefa Girona, Montse Guardiola, et al.
   Clinical Chemistry and Laboratory Medicine 2008;46:453-457.
3) Protease inhibitor-associated dyslipidemia in HIV-infected patients is strongly influenced by the APOA5 -1131T>C gene variation.
   Montse Guardiola, Raimon Ferré, et al.
   Clinical Chemistry 2006;52:1914-1919.

FUNCTIONAL STUDIES:

Paradoxically, while there was growing evidence on the influence of APOA5 on TG levels, very little was known about its physiological role. Hence, we designed two studies directed towards gaining an insight into the functionality of the gene. The results were presented in the following articles:

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4) La grasa saturada en la dieta disminuye la expresión hepática de APOA5 en ratones deficientes en APOE *[Saturated fatty acid diet decreases the expression of APOA5 in APOE deficient mice].*
   Montse Guardiola, Mònica Tous, et al.
   Clínica e Investigación en Arteriosclerosis 2007;19:22-29.
5) The APOA5 gene is expressed in human intestinal tissue and responds to fatty acid and fibrate exposure *in vitro*.
   Montse Guardiola, Adriana Alvaro, et al.
   Submitted Biochim et Biophys Acta 2008.

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INCREASED CONCENTRATIONS OF CIRCULATING VITAMIN E IN CARRIERS OF THE APOLIPOPROTEIN A5 GENE - 1131T>C VARIANT AND ASSOCIATIONS WITH PLASMA LIPIDS AND LIPID PEROXIDATION.

Sundl I, Guardiola M, Khoschsorur G, Solà R, Vallvé JC, Godàs G, Masana L, Maritschnegg M, Meinitzer A, Cardinault N, Roob JM, Rock E, Winklhofer-Roob BM, Ribalta J.

J Lipid Res 2007;48(11):2506-13.

ISBN:978-84-691-9478-2/DL:T-19-2009

When we started the first study, there had not been any previous publications on the association of the APOA5 gene in relation to a detailed lipid profile (complete lipid, lipoprotein and apolipoproteins), nor in relation to the vitamins carried by the lipoproteins, especially vitamin E (a fat-soluble molecule strongly linked to TRL metabolism, and with a well-documented lipid antioxidant function). We genotyped a population sample of 297 male non-smokers from France, Austria and Spain. This population sample was part of the *Vitage* study aimed at identifying lipids and vitamin changes in the course of ageing. The population sample had 2 main characteristics: strict selection criteria for the absence of disease, and detailed lipid and vitamin profiles. We hypothesized that, in this group of healthy subjects, the confounding effect of metabolic factors on the genetic influence would be minimal and would help highlight the real influence of the APOA5 gene.

The frequency of the minor allele (-1131C) among the healthy men was 0.08, which is similar to that in many Caucasian populations. In our population sample of healthy men, the presence of the C allele was associated with significant alterations in several lipid-related parameters. Carriers of the C allele presented with 15% higher circulating TG levels, mainly due to an increased content in VLDL fraction; higher cholesterol levels in VLDL and IDL fractions; and almost 9% higher circulating fatty acids, without any difference in fatty acid intake between genotypes. They also presented with higher amounts of circulating apo B levels which is a marker of the number of apo B-containing lipoprotein particles and, as such, had higher circulating levels of VLDL and LDL lipoproteins. The influence of APOA5 genetic variant on lipid metabolism remained following adjustment for the BMI, age and dietary intake.

Vitamin E consists of 8 different isoforms of tocopherols and tocotrienols. We found that only plasma α-tocopherol (but not γ-tocopherol, LDL α- and γ-tocopherol, nor total vitamin E from buccal mucosa cells) was significantly increased by 9.1% in C allele carriers compared with homozygote T-carrying subjects, even after adjustment for BMI and age, but not when adjusted for total lipid levels. This indicated that TG and vitamin E increased in similar proportions. The adjustment for cholesterol or total lipids is appropriate since vitamin E is closely associates with lipids and depends on lipid or lipoprotein metabolism for delivery to tissues (253). Since vitamin E is a well-documented antioxidant molecule, we also analyzed the effect of APOA5 genotype on antioxidant status, as measured by plasma malondialdehyde concentration (a marker of the lipid peroxidation rate). We observed no statistically significant differences with respect to genotype, and confirmed that TG and vitamin E increased proportionally.

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Conclusions from Study 1: The results demonstrated that TG elevation associated with the -1131C variant was mostly due to an increase in the VLDL fraction; both at the level of TG enrichment and particle number, as suggested by increased VLDL-apo B. Increases in VLDL resulted in increased amounts of VLDL-associated vitamin E, which did not confer increased antioxidant protection.

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THE APOLIPOPROTEIN A5 GENE -1131T-->C POLYMORPHISM AFFECTS VITAMIN E PLASMA CONCENTRATIONS IN TYPE 2 DIABETIC PATIENTS.

Girona J, Guardiola M, Cabré A, Manzanares JM, Heras M, Ribalta J, Masana L.

Clin Chem Lab Med. 2008;46(4):453-7.

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The first population sample studied was specifically selected to be free of any known disorder. We wished to confirm our results in diabetic patients since these patients present with atherogenic dyslipidemia. The defects are defined mainly by alterations in TG metabolism (high TG levels, low HDLc levels, presence of sdLDL particles) as well as increased oxidative stress. This latter status is believed to play an important role in the development of type 2 diabetes (DM2) with major attention being focused on lipid oxidation, specifically on that of LDL particles. Data we generated on these patients included a complete lipid, lipoprotein and oxidative status profile as well as vitamin E levels and parameters related to LDL oxidation.

We genotyped the -1131T>C polymorphism in 169 DM2 patients diagnosed in accordance to the American Diabetes Association criteria (274). Patients with type 1 diabetes mellitus, secondary diabetes mellitus, morbid obesity (BMI >40 kg/m^2), familial hypercholesterolemia, diabetic retinopathy and/or nephropathy, malignancy, liver disorders, acute or chronic inflammation and smokers (at least one cigarette in the previous year), were excluded. Among the 169 diabetic subjects, 73 had evidence of atherosclerosis as indicated a clinical history of coronary heart disease, stroke or peripheral vascular disease involving at least one significant arteriosclerotic plaque (>40% stenosis) measured by carotid and femoral echo-Doppler, and/or an ankle brachial index ≤0.9 or ≥1.3.

We observed that the minor allele frequency was similar to that described in other studies conducted in Caucasian populations (approx. 0.07). There were no statistically significant differences between carriers and non-carriers of the minor allele with respect to BMI, blood pressure, glucose, glycated hemoglobin, and presence of atherosclerosis. Supporting the results described in our previous study, we found that subjects carrying the rare allele at position -1131 had a significant increase of 21% in circulating TG levels, mainly due to differences in VLDL and HDL fractions. Also, they had 8% higher plasma apo A-I levels and 12% higher apo C-III levels. These results were confirmed even when adjusted for the hypolipidemic treatment that the patients were receiving.

Lipid oxidation is a crucial mechanism in the evolution of diabetes, as well as atherosclerosis. Among the parameters measured (vitamin E, LDL dienes formation, lipoperoxides, oxidized LDL, oxidized LDL antibodies) in assessing oxidative status, we only found significant differences in vitamin E levels in relation to genotype. The differences remained statistically significant when normalized for cholesterol, but not when normalized for TG levels. In these diabetic patients, the carriers of the C allele had 11.3% higher concentrations of vitamin E compared with TT subjects, and the

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frequency of rare allele carriers was higher among the group of subjects with higher circulating vitamin E concentrations (2.6-fold higher).

Conclusions from Study 2: We confirmed that TG levels increases associated with the -1131C allele are due to increased VLDL particles; the influence of this genetic variant being more important in diabetic patients than in healthy subjects.
We confirmed, as well, that the resultant increase in circulating vitamin E levels was not associated with changes in oxidation status.

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PROTEASE INHIBITOR-ASSOCIATED DYSLIPIDEMIA IN HIV-INFECTED PATIENTS IS STRONGLY INFLUENCED BY THE APOA5-1131T->C GENE VARIATION.

Guardiola M, Ferré R, Salazar J, Alonso-Villaverde C, Coll B, Parra S, Masana L, Ribalta J.

Clin Chem. 2006;52(10):1914-9.

ISBN:978-84-691-9478-2/DL:T-19-2009

Hypertriglyceridemia is the main side-effect of a number of drugs, including retinoic acid and protease inhibitors (PI). Over recent years there has been increasing interest in assessing an individual's genetic inheritance in relation to response to certain therapeutic agents. We hypothesized that APOA5 is likely to exacerbate such predisposition to hyperTG. We investigated the possible modulatory effect of APOA5 genetic variation (-1131T>C) on the well-described dyslipidemic effect of PI which was being used as antiretroviral therapy in our hospital. The study sample contained 229 HIV-positive patients, >18 years of age, without AIDS-related opportunistic diseases at the commencement of the study and, from whom, we had clinical and biochemical data from five years of follow-up. The lipid parameters were compared in two sub-groups: those receiving PI (n=148) and those not receiving PI (n=81). Only the results from the first 3 years of treatment were published because there were fewer patients with detailed lipoprotein profiles at years 4 and 5 of follow-up. This is probably because, in the past, it was not usual to analyze lipid parameters in HIV patients, or were measured only in those patients with the most evident dyslipidemia.

Our results indicated that, although all patients at baseline (commencement of therapy) had similar plasma TG levels, the group of patients treated with PI and carriers of the -1131C allele had significantly higher TG levels following treatment (43% higher after one year, 38% increase after two years, and 44% after three years) relative to non-carriers of this allele. These values remained significant even after adjustment for age, gender, BMI, and the presence of lipodystrophy. A similar effect was observed for total cholesterol levels. C allele carriers treated with PI had 16% higher cholesterol levels after one year of treatment, 21% increase after two years and 20% after three. We also found a tendency towards lower HDL cholesterol levels among the group of C allele carriers treated with PI, but which did not reach statistical significance. Of note is that, after 3 years of treatment, patients treated with PI and carriers of the APOA5 -1131C allele had a dyslipidemic profile that was associated with a significant difference in the atherogenic index, as measured by the total cholesterol/HDL cholesterol ratio among genotypes i.e. HIV patients with the C allele treated with PI had a 78% increase in the atherogenic index compared to patients who were wild-type allele carriers.

Conclusions from Study 3: This is the first published pharmacogenetic study assessing the hyperTG effect of the -1131C promoter variant of APOA5 gene and the response to highly active antiretroviral therapy. The magnitude of such hyperTG is greater than that of healthy subjects and of diabetic patients and confirms the notion that the effect of APOA5 largely depends on the metabolic background of the subject (data summarized in Figure 14).

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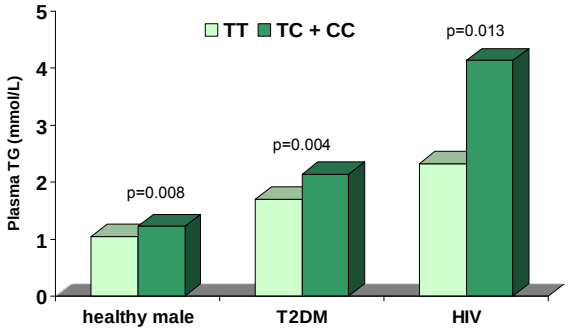

Figure 14. Plasma TG levels among study subjects segregated according to -1131T>C variant of the APOA5 gene. Healthy male carriers of the C allele present with a 15% increase in TG levels compared to TT subjects; the increase being higher in type 2 diabetic patients (21%) and greater in HIV-infected patients (44%).

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LA GRASA SATURADA EN LA DIETA DISMINUYE LA EXPRESIÓN HEPÁTICA DE APOA5 EN RATONES DEFICIENTES EN APOE

[Saturated fatty acid in the diet decreases the hepatic expression of APOA5 in APOE deficient mice]

Guardiola M, Tous M, Vallvé JC, Rull A, Masana L, Joven J, Ribalta J.

Clin Invest Arterioscl. 2007;19(1):22-9.

ISBN:978-84-691-9478-2/DL:T-19-2009

Study 4 was aimed at investigating whether the APOA5 gene expression was responsive to dietary fat, a major determinant of TG homeostasis. We took advantage of an animal model, the APOE knock-out (KO) mouse. The model (KO-APOE) would enable us to study not only APOA5 expression in relation to diet but also to explore its possible relationship with atherosclerosis.

Studying the development process of atherosclerosis, the pathogenic mechanisms, and the genetic-environmental factors involved in humans presents difficulties that can be partially overcome using animal models. Mice are good experimental animals since they are small, easy to use, with a short generation time and cheap. However, normal mice do not usually develop atherosclerosis because their plasma cholesterol levels are low and the cholesterol is carried mainly in HDL particles. The genetically modified APOE deficient mice develop higher cholesterol levels, distributed mainly among lipoproteins with particle sizes corresponding to VLDL or IDL. The plasma TG levels are not affected and their lipid metabolism resembles human. As a result, APOE deficient mice can quickly and reproducibly develop arteriosclerosis, even when fed a normal diet. When they receive fat- and cholesterol-rich diets, their cholesterol levels in plasma and the atherogenesis process increase proportionally and, as such, have been used extensively to study arteriosclerosis.

We analyzed the hepatic APOA5 gene expression in 72 APOE-KO mice separated into different groups depending on the diet received: standard chow (control animals), saturated fat-rich diet (20% palm oil) alone, diet supplemented with 0.25% cholesterol. In each group, the mice were sacrificed at different ages (16, 24, and 32 weeks). This study design enabled us to analyze the effect of diet and age on the expression of APOA5, and to relate these effects to circulating inflammation markers. We also measured the extent of atherosclerotic lesion area in the aorta.

We found that after administration of saturated fat-rich diet, alone or supplemented with cholesterol, APOE deficient mice had significant increase in circulating cholesterol levels relative to control mice (fed with standard chow), but without differences in relation to circulating TG levels. However, the amount of lipids (cholesterol and TG) contained in the liver were higher when mice were fed the atherogenic diets. The administration of such diets also affected the extent of aorta lesion as well as degree of steatosis; both of which were greater relative to control mice.

Adding palmitic acid to the diet promoted the decrease in APOA5 hepatic expression by 75% in the group of mice aged 16 weeks, 35% in those aged 24, and 45% in those aged 32, compared to that in control mice. Nevertheless, the supplementation with 0.25% cholesterol counterbalanced the effect of palmitic acid on APOA5, and which

ISBN:978-84-691-9478-2/DL:T-19-2009

was similar to that of control mice. These effects were also found in mice at different ages.

Subsequently, we re-grouped the data on all the mice in relation to age at which they were sacrificed, and irrespective of the type of diet received. We found that APOA5 expression in liver increased significantly with age, and this effect was also found when evaluating each diet-group separately.

Correlation analyses were performed to assess whether the expression differences due to diet were related with any lipid or atherosclerotic parameter measured in the study. We found that hepatic APOA5 gene expression was not related with any of the lipids measured in these mice, but was positively correlated with the extent of aortic lesion and with plasma concentrations of monocyte chemotactic protein-1 (the key inflammatory marker in mice). The positive correlation was found only in those animals fed with fat-rich diet, and was negatively correlated in mice fed with normal chow, or with the diet supplemented with cholesterol.

Conclusions from Study 4: The results obtained demonstrated, for the first time, that the expression of APOA5 could be modified by the type of diet consumed and, as a result, there is a different behavior in relation to inflammatory parameters and arteriosclerosis. However, these results were obtained in an animal model that had been genetically modified (APOE deficient mice) having a defect in the TG secretion pathway in hepatocytes. This effect of diet on APOA5 gene expression needs to be explored in depth in humans.

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THE APOA5 GENE IS EXPRESSED IN HUMAN INTESTINAL TISSUE AND RESPONDS TO FATTY ACID AND FIBRATE EXPOSURE IN VITRO.

Guardiola M, Alvaro A, Vallvé JC, Rosales R, Solà, R, Girona J, Duran P, Esteve E, Masana L, Ribalta J.

Biochem et Biophys Acta (submitted 2008)

ISBN:978-84-691-9478-2/DL:T-19-2009

Based on the hypothesis that apo A-V may participate in the synthesis and/or secretion process of TG-rich lipoproteins, we designed Study 5 to evaluate APOA5 gene expression in small intestine.

RNA from tissue samples from different parts of the human small intestine obtained from *BD Biosciences Clontech* were used to assess whether apo A-V is present in the intestine which, together with the liver, would be responsible for TG-rich particle formation. Also, to assess whether APOA5 expression is affected by dietary fatty acids or whether pharmacologic agents may affect its expression rate, we used the human intestinal cell line TC-7 (a sub-clone of the well-characterized Caco-2 cells) incubated with different types of fatty acids at different concentrations (long-chain fatty acids: 50, 100, 200 and 300μM; short-chain fatty acids: 2 and 5 mM) over 24h. A synthetic PPARα agonist was tested at 100μM incubation concentration over 24h. We also measured the expression of APOB and MTP genes (critical in the TRL synthesis and secretion process) and APOC3 gene (also important in regulating TG metabolism) which have been proposed as playing antagonistic roles in relation to the APOA5 regulation of TRL.

We detected APOA5 expression in the intestine. Our results indicated that the studied genes were differently expressed among the different parts of the human intestine i.e. APOA5 is expressed mainly in duodenum and colon, while APOB is highly expressed in duodenum, jejunum and ileocecum, but not in colon. Similarly, MTP is highly expressed in duodenum and jejunum, but not in colon, while APOC3 is mainly found in jejunum. Correlation analyses indicated an inverse relationship between APOA5 and APOC3, but which did not quite reach statistical significance ($r = -0.555$).

Having been able to detect APOA5 gene expression in the intestine, albeit at very low levels, we proceeded to design experiments in which we incubated the human intestinal TC-7 cell line with different types of fatty acids. We observed that APOA5 expression is down-regulated by mono- and polyunsaturated fatty acids, while saturated fatty acids up-regulate APOA5 expression. The effect of saturated fatty acids vs. the effect of polyunsaturated on APOA5 expression was significantly different. Of note was that the short-chain fatty acid butyrate increases APOA5 expression almost 4-fold relative to control cells (no butyrate in the incubation), while APOB is down-regulated by increasing butyrate acid concentrations. When incubating TC-7 cells with a PPARα agonist (Wy 14,643) we observed, as expected from other published studies, that APOA5 expression was increased by 60%, while the expression of APOB, MTP and APOC3 were decreased by 50, 30 and 45%, respectively.

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Conclusions from Study 5: For the first time in the published literature, we identified APOA5 in tissue derived from human small intestine. We demonstrated that the expression of APOA5 can be modulated by dietary and pharmacological stimuli, and that its concentration is inversely correlated with the APOC3 gene.

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Discussion

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Discussion

Circulating TG levels have been repeatedly proposed as an independent risk factor for cardiovascular disease; a disease that is currently one of the main causes of death in industrialized countries. Plasma TG levels are strongly influenced by environmental (diet, lifestyle habits...) and by genetic factors. The main objective of this thesis was to contribute to the knowledge of TG metabolism modulation, through the study of APOA5 gene which has been described as an important gene in lipid metabolism, chiefly in the regulation of TG levels. We have hypothesized that its influence may affect other aspects tightly linked to TG metabolism, such as vitamin transport and medication-induced hypertriglyceridemia. Since apo A-V modulates TG levels, we also hypothesized that its expression would be modulated by dietary elements and, as such, should be identifiable in the intestine.

As mentioned earlier in the Introduction section, distinct polymorphisms in the APOA5 have been recently identified as having strong influence on TG metabolism leading to increases the plasma levels in a wide range of human populations, and with some differences with respect to ethnicity and gender (250). Using association studies, our purpose was to contribute to the knowledge of APOA5 on lipid metabolism. For this we evaluated the -1131T>C promoter variant, which has been widely used as a genetic marker, in populations with different metabolic background with well-characterized lipid and lipoprotein profiles. Also, we conducted pharmaco-genetic studies to detect potential interactions between the gene and drugs affecting TG levels.

Initially, we confirmed the association of the gene with parameters of the lipid metabolism by analyzing the prevalence and effect of the rare allele in two populations with different metabolic background (healthy males and patients with type 2 diabetes) in whom comparable information was available: complete lipid, lipoprotein and apoprotein profiles together with detailed oxidative status parameters.
Results from both studies confirmed what has been published by others i.e. APOA5 significantly alters parameters related to TG metabolism even in healthy individuals. We described that the elevations of plasma TG are due to an elevation of TG content of the VLDL fraction.
In general, an increase in circulating TG levels result from an increase in the hepatic synthesis or by inhibition of the hydrolytic activity by LPL. Although the precise function of apo A-V has not, as yet, been clarified, the published literature (153, 156, 165) indicates that it may affect triglyceride-rich lipoprotein (TRL) lipidation, synthesis and

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secretion processes as well as its catabolism and the clearance of its remnants from circulation. Therefore, it is likely to be involved in all important steps of TRL metabolism. From our results we can speculate that synthesis and clearance mechanisms may be involved in the TG-elevating effect. This is based on our findings, firstly, that healthy male carriers of the C allele have higher circulating apo B levels which is indicative of the amount of circulating TRL particles. This can be the result of increased synthesis, decreased catabolism, or both. The increased concentrations of FFA may support the possibility of increased synthesis since they are known to stimulate VLDL synthesis and secretion in the liver. Secondly, in both populations we found increased levels of apo C-III in plasma, which is an inhibitor of the TG-hydrolytic activity of LPL. Increased TG associated with the C allele is accompanied by increased apo C-III. Since the TG/apo C-III ratio is not different between genotypes, this would appear to be a consequence of the high TG, rather than a cause. Finally, the higher levels of apo B-containing lipoproteins in healthy subjects who are carriers of the -1131 C allele could probably explain why we detected an association with cholesterol levels in VLDL and LDL fractions when not differences between genotypes were observed with respect to total cholesterol levels.

In agreement with our results are several publications describing the association with lipid metabolism, most of them showing the significant effect on TG levels, and with some discrepancy when evaluating other lipid metabolism parameters such as total cholesterol, HDL cholesterol, and circulating apolipoproteins levels. A certain discrepancy may be expected since the studies were performed in different population samples. The probably indicates, as has been proposed by other authors, that apo A-V influence on lipid parameters is highly dependant on other factors such as ethnic origins, metabolic background, gender, treatment, etc.

In support of our observations are the results from the Framingham Study population (101) describing significant associations with increased total cholesterol and TG levels, cholesterol from LDL particles, presence of remnant particles, higher amount of intermediate VLDL particles and smaller LDL size, and increased circulating apo B and apo A-I levels.

The apo B levels are indicative of the amount of circulating particles which likely reflect the influence of apo A-V on synthesis and secretion of the TRL or, alternatively, an influence on their clearance from circulation; both aspects being influenced by APOA5 gene. With respect to the association between APOA5 and particle diameter, it is likely to be a consequence of the effect that TG have on CETP and, therefore, not directly linked to APOA5.

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Other studies have published significant effects between APOA5 gene variants and HDL-cholesterol levels (234, 183, 190). It is reasonable, taking into account that the mechanism by which apo A-V mediates changes in TG concentrations could also affect HDL metabolism due to the well-documented strong inverse correlation existing between TG and HDL levels (see Introduction section) to detect differences with respect to genotype and circulating apo A-I levels. We have also described an association with apo C-III levels (indicative of the degree of inhibition of TG hydrolysis in circulation) resulting in increased TG levels, and with a higher concentration of total fatty acids related to increased VLDL production rate by the liver. Therefore, the effect on TG and apo B levels may be directly linked to APOA5, while the association with apo A-I, particle size, HDL and apo C-III concentrations are probably a consequence since, on adjusting for TG in multivariate analysis, most of these associations disappear.

We have been the first to describe that this effect on TG metabolism is accompanied by increased circulating vitamin E concentrations (in both our study populations with different metabolic backgrounds) but which do not relate to any differences in oxidative status.

Vitamins are essential organic micronutrients required at quite low concentrations for various physiological functions such growth and the nutritional balance that defines health. Vitamins are divided into two groups depending on their solubility: fat-soluble (A, D, E, K) and water-soluble vitamins (B, C).

Fat-soluble vitamins are absorbed from the diet and, due to their hydrophobic nature, require proteins or lipoproteins for their transport in circulation. This implies that fat-soluble vitamins are strongly correlated with serum lipid parameters and which led us to hypothesize that genetic markers associated with lipid parameters would also be associated with fat-soluble vitamin levels. There are few studies describing lipid-related genetic markers associated with levels of fat-soluble vitamins in circulation. When we commenced our study, only Ortega et al (276) had described an association between apo E genotype and circulating vitamin E levels in Spanish children. Since our study was published, Borel et al (277) described a significant association between SNPs in some genes related to lipid metabolism (APOA4, APOB, APOE, LPL, SR-BI) on vitamin E and carotenoids levels in subjects displaying moderate risk for cardiovascular disease.

Our population sample composed of healthy males was part of the VITAGE Project (Contract QLK1-CT-1999-00830) (278) which was designed to evaluate the status and

ISBN:978-84-691-9478-2/DL:T-19-2009

metabolism of vitamin A, vitamin E, and carotenoids during ageing; the rationale being that ageing is often associated with degenerative diseases such as certain cancers, neurological disorders and cardiovascular diseases. The main objective was to provide evidence regarding changes in status, metabolism, and function of fat soluble vitamins during non-pathological ageing. Thus, in our healthy population we could also evaluate the potential association between APOA5 gene polymorphism and other fat soluble vitamins (vitamin A, α-carotene, β-carotene, lycopene, lutein, criptoxanthine and zeaxanthine), as well as tocopherols (data not published). Among the fat-soluble vitamins studied, the APOA5 gene was associated only with α-tocopherol. There was no significant effect with respect to any other vitamins including the vitamin E isoform γ-tocopherol. Carriers of the C allele had 9.1% higher vitamin E levels in healthy men and 11.3% higher levels among diabetic patients.

We speculate that APOA5 is not associated with the other fat-soluble vitamins studied because vitamin A is transported with retinol binding protein which is not strongly linked to lipoprotein metabolism. The other vitamins are found at very low concentrations in circulation (less than one μmol/ L) and, as such, it is possible that there may need to be great differences for the differences to reach statistical significance. However, it cannot be ruled out that such association may exist in a larger population sample.

Vitamin E correlates strongly with TG metabolism which is regulated by APOA5 gene and this may point to why APOA5 may be having an impact on plasma vitamin E plasma levels. Vitamin E is a fat-soluble antioxidant molecule comprising eight different isoforms, including α-, β-, γ-, and δ- tocopherols and tocotrienols. The vitamin E isoforms are absorbed equally with dietary lipids and enter the circulation via the chylomicrons from which the action of LPL is to transfer the vitamins to extra-hepatic tissues in the course of TG hydrolysis, and with the phospholipid transfer protein exchanging them between different lipoprotein classes. When the vitamins reach the liver, the α-tocopherol transfer protein preferentially recognizes the α-tocopherol forms incorporates them into VLDL particles. The other vitamin E forms are excreted into bile, metabolized and excreted into urine (see Figure 15). Consequently, α-tocopherol, which also shows the highest biologic activity, is the major form of vitamin E in human plasma and tissue. Hence, from what we have summarized on APOA5 (see Introduction section), the most important steps in vitamin E metabolism may be influenced by APOA5 gene variants.

We found that the hypertriglyceridemic effect of APOA5 is explained, mainly, by an effect on VLDL fraction. APOA5 also induces higher levels of α-tocopherol, which is the isoform of vitamin E preferentially selected in the liver to enter circulation incorporated within VLDL particles.

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ROLE OF THE APOA5 GENE IN LIPID AND FAT-SOLUBLE VITAMIN METABOLISM
Montserrat Guardiola Guionnet
ISBN:978-84-691-9478-2/DL:T-19-2009

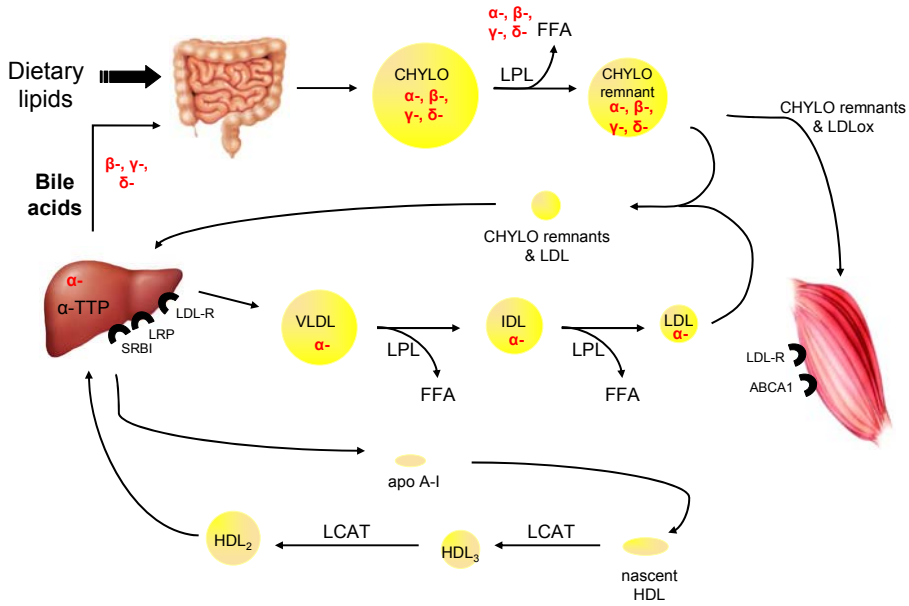

Figure 15. Representation of vitamin E metabolism; α-, β-, γ-, δ- are different vitamin E isoforms; α-TTP= alpha tocopherol transfer protein.

To summarize, C allele carriers have higher amounts of circulating TG levels and number of TRL particles which have been linked to a greater cardiovascular risk. Conversely, higher amounts of vitamin E have been inversely correlated with mortality from ischemic heart disease (279). This is perhaps explained by the best-known function of vitamin E which is to scavenge reactive oxygen species, i.e. inhibiting lipid peroxidation, a physiological phenomenon well-documented as being directly involved in cardiovascular disease development.

We expected to find differences in lipid peroxidation parameters that had been measured in our two population samples (in the healthy population sample we had data from plasma malondialdehyde, LDL dienes formation, F2 isoprostanes, plasma total peroxides, glutathione peroxidase and urinary deoxyguanosine (data not published). In the diabetic population sample we had data from LDL dienes formation, lipoperoxides, LDLox and antibodies for LDLox-. In both populations the increased vitamin E concentrations associated with the C allele did not alter the individual antioxidant capacity. Our results are not in accordance with those of Jang et al (92) who found that C allele carriers had significantly higher levels of CRP, F2 isoprostanes and DNA damage. The population reported by Jang et al included smokers, while our population samples were composed entirely of non-smokers, a situation that significantly affects the oxidative status of the subject. Also the different genetic backgrounds, as evidenced by the marked differences in the frequency of this particular polymorphism

ISBN:978-84-691-9478-2/DL:T-19-2009

(approx 0.08 vs. 0.28 in Spanish and Korean populations, respectively), is likely to be a major factor in explaining the differences observed.

The lack of differences in the antioxidant capacity in relation to higher vitamin E levels, may be explained by the strong linkage that exists between vitamin E and TG. There is an increase in the amount of the oxidative substrate (TG) but also an increase in the amount of the antioxidant (vitamin E). This was confirmed by normalizing vitamin E for different lipid parameters and the lipid/vitamin E ratios remaining the same in plasma as well as the LDL fraction.

The differences in the absolute amounts of circulating vitamin E may be important due to the various functions of vitamin E, independently of their antioxidant capacity. For example, vitamin E also acts as an intracellular trafficking signal, as a genetic regulator modulating the expression of genes involved in tocopherol uptake and degradation (α-TTP, CYP3A, …), on genes involved in lipid uptake and arteriosclerosis (SR-BI, CD36, …), on genes modulating extra-cellular proteins such as metalloproteinases, on genes involved in cellular adhesion and inflammation (ILs, ICAM1, …), and also on genes of cellular signaling and cell cycle (PPARγ, cyclines, …). Finally, vitamin E also contributes to anti-inflammatory and immunological mechanisms (reviewed by Azzi A. (280, 281). Is likely that this findings may involve, as has been recently published, apo A-V with inflammation (167, 168) and, as such, contributing via different mechanisms, to the development of cardiovascular disease.

From the literature and from our own results (182), -1131T>C gene variant predispose to hyperTG and that the higher the stimulus, the higher the response of the variant. We confirmed these observations with the healthy as well as the diabetic patient sample. One potent inducer of hyperTG is the exposure to certain drugs/medications such as protease inhibitors. Hence, following our study of APOA5 gene variants in relation to lipid metabolism, we hypothesized that the APOA5 -1131 T>C gene marker would influence TG metabolism in HIV-infected patients receiving anti-retroviral therapy. We explored this possibility in a pharmaco-genetic study involving 229 HIV-infected patients followed-up over 5 years. When we initially analyzed the results of the genotyping, the APOA5 variant did not appear to have a significant effect on TG metabolism. However, when the subgroup of patients receiving protease inhibitors (PI) was considered separately, it became evident that carriers of the -1131 C allele were at much higher risk of developing severe and sustained hypertriglyceridemia, leading to the worsening of their lipid profile. Of note is that all patients had similar values for TG and cholesterol levels when commencing their treatment but, following anti-retroviral

ISBN:978-84-691-9478-2/DL:T-19-2009

treatment for one, two and three years, we detected significant differences among C allele carriers treated with PI, compared to the rest of the patient group. Numerically, this implies that C allele carriers have at the end of 1 year of PI treatment, a mean of 43% higher TG levels and 16% higher total cholesterol levels. After 3 years of treatment these levels are 44% and 20% of TG and cholesterol, respectively. Of note is that the patients carrying the C allele but not receiving PI therapy had lipid levels that were similar to carriers of the wild type allele.

In general, elevated lipid levels may be explained by increased lipoprotein synthesis, or the decrease in lipoprotein catabolism, or a combination of both. The protease inhibitor-related hyperlipidemic effect is, in part, mediated by a direct interaction between the medication and the sterol regulatory element-binding protein (SREBP); one of the regulatory elements in the expression of certain genes involved in the lipid metabolism, resulting in an increased lipoprotein synthesis (282). APOA5 is one of the target genes for the SREBP, but the mechanism by which it strongly regulates TG levels is still unclear. Apo A-V has been shown to influence VLDL synthesis and secretion in the liver and, as well, to stimulate TRL catabolism via the clearance of remnants from the circulation. As such, the likely alteration in the apo A-V protein activity due to the presence of the -1131T>C variant (181) may be responsible, at least in part, for the hyperlipidemic phenotype observed in the HIV patients treated with PI (see Figure 16 for schematic representation of the hypothetical pathway).

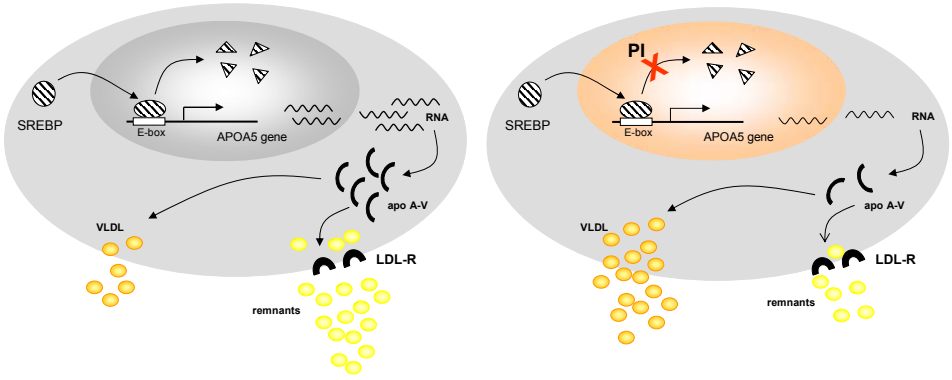

Figure 16. SREBP transcription factors recognize the response element in APOA5 gene promoter sequence and inhibit its transcription. The amount of SREBP in the nucleus is controlled by specific cleavage, and enabling an appropriate apo A-V production rate. Once in cytoplasm, apo A-V could control VLDL synthesis and secretion, or stimulate the uptake of circulating remnants. Protease inhibitors bind to the SREBPs preventing their specific cleavage. Hence, we postulate that SREBPs are accumulated in the nucleus during the anti-retroviral therapy and suppress longer APOA5 gene transcription. This could result in a greater VLDL synthesis and secretion process, or to a lower stimulation of remnant uptake. The outcome would the development of the dyslipidemia observed in HIV-patients treated with PI.

ISBN:978-84-691-9478-2/DL:T-19-2009

It is well documented that HIV-patients receiving anti-retroviral therapy have an increased risk for cardiovascular disease due to the metabolic complications associated with their treatment (283). Because the atherogenic ratio (total cholesterol/HDL-cholesterol) in our study sample reached the highest quintile of risk among the C allele carriers treated with PI, we decided to assess whether the presence of the atherogenic dyslipidemia due to the APOA5 polymorphism was indeed involved in raising the cardiovascular disease risk. We measured the endothelial intima and media thickness (IMT) in the aorta of these patients at two different time-points of follow-up. The IMT has been widely described as a surrogate measure of the atherosclerosis burden (284). We did not find any statistically significant differences in relation to genotype (unpublished results) but we believe that this may be explained on the measurements having been conducted within too short a follow-up period. Our highly significant findings of association between APOA5 with the atherogenic lipid profile had been observed after three years of treatment and, as such, perhaps significant differences in IMT would, as well, become more evident after a similar period of treatment of three years or more .

In support of this assumption are the findings in a subgroup of the Framingham study population of APOA5 gene variations being significantly associated with carotid artery IMT; the effect being influenced by obesity (174). However, in a smaller population composed of FCHL families, no significant association was found between IMT values and -1131T>C or S19W APOA5 gene markers (172). Further, with respect to the negative association between APOA5 gene marker and the sub-clinical atherosclerosis measured in our HIV population sample, we need to take into account that the relationship between A5 and the CVD risk has been studied over the long-term, and the results are far from consistent (see Introduction section).

A similarly designed study involving HIV patients was published in 2007. Several selected genotypes from different key genes for the lipid metabolism (ABCA1, APOA5, APOC3, APOE, CETP, LPL, MTP, and TNF among others) were studied in a group of HIV patients treated with antiretroviral therapy for almost five years. The objective was to identify associations between the lipid parameters and genetic variants. The conclusions from the study were that APOA5 gene, together with variants in ABCA1, APOC3, APOE and CETP genes, contributed to the dyslipidemia linked to the antiretroviral therapy (242).

To summarize: all classes of drugs/medications used as antiretroviral treatment have been associated with atherogenic changes in the patients' lipid profile (285). Even though antiretroviral therapy has been associated with other well-documented risk

ISBN:978-84-691-9478-2/DL:T-19-2009

factors for cardiovascular disease, the accumulation of TRL remnant particles (286) over the extended chronic administration of this treatment and the presence of a key genetic variant would exacerbate this pro-atherogenic status. Our results, and their confirmation, would suggest that the measurement of APOA5 be used as a marker for a patient's predisposition to hyperlipidemia secondary to treatment with protease inhibitors.

The role of APOA5 as a modulator of the metabolic derangements associated with drug treatment is supported by another recent pharmaco-genetic study evaluating APOA5 gene variants and their effects on fenofibrate treatment.

Fibrates have been the main option over several decades for the treatment of hypertriglyceridemia. The molecular mechanisms underlying the lipid lowering effects are still unclear but it is understood that fibrates activate one isoform of the nuclear receptor peroxisome proliferator-activated receptor (PPARα) that modulates the expression of genes. APOA5 is one of the target genes for PPARα and, in hepatocytes, its expression is stimulated by PPARα agonists (Wy14643, fenofibrate or GW9003). As such, of interest would be the action of fibrates on TG metabolism and their modulation by APOA5 gene variants. This was the main objective of the study by Lai et al (224). They reported an association between 2 common variants in the APOA5 gene (-1131T>C and 56C>G) and TG and HDL-cholesterol plasma concentrations (at baseline and postprandial) in response to fenofibrate treatment in 791 subjects from a general population (GOLDN Study). Their results showed that subjects with APOA5 -1131T>C genotype have no significant differences in response to fenofibrate therapy. Conversely, carriers of the G allele of the 56C>G genotype have a greater ability to decrease TG and increase HDL-cholesterol in response to the fibrate therapy. As such, the APOA5 56G carriers would benefit more from fenofibrate treatment.

Despite all the evidence showing association between APOA5 and the predisposition to hyperTG, very little is known about the underlying function. This is aggravated by the overall data on plasma apo A-V and TG levels. Most evidence from population association studies and animal model data show a consistent inverse correlation between apo A-V and circulating TG concentrations but which, on occasion, has been shown to be positive or even non-existent. Different possibilities have been invoked to explain these contradictory findings. The lack of sensitivity of the measurement of very

ISBN:978-84-691-9478-2/DL:T-19-2009

low concentrations of apo A-V in circulation may be responsible of this disagreement but also the possibility that apo A-V may be recycled (157) would suggest that the great differences between the amount of protein and the lipoproteins is not so critical. Also apo A-V may not be the key factor relating to the TG levels observed (220, 225, 254) since these associations may be explained by a strong linkage disequilibrium (LD) with other genetic variants of the APOA1/C3/A4 gene cluster. Finally, the results from animal models should be interpreted with caution since different regulators may be specific for different species. However, we and others, believe that the role of apo A-V may be identified within cells, rather than in plasma.

We planned two studies to explore this option, but which did not focus on its effect on TG levels. We then proceeded to investigate the function of APOA5 using animal models and cell culture experiments.

In 2006 we studied the effect of an atherogenic diet on hepatic APOA5 expression using the APOE deficient mouse model which, when administered a Western-type diet, develops atherosclerosis.

We described, in the article published in *Clínica e Investigación en Arteriosclerosis* that the mice, when fed a diet rich in saturated fat, had a 50% decrease in the hepatic expression of APOA5 compared to wild-type mice and that the effect was attenuated when the diet was supplemented with cholesterol.

The exact mechanism by which dietary fatty acids or cholesterol could modulate the hepatic expression of APOA5 gene in APOE deficient male mice is unclear, but it is consistent with the regulatory role that certain nuclear transcription factors exert on APOA5 gene i.e. most of them are activated by either fatty acids, or cholesterol, or both. As we have seen earlier in the Introduction section, APOA5 gene expression is regulated by many transcription factors known to affect TG metabolism (287) such as peroxisome proliferator-activated receptor (PPARα), sterol regulatory element binding protein (SREBP), liver X receptor (LXR), hepatocyte nuclear factor 4-α (HNF-4α) and retinoic acid receptor-related orphan receptor-α (RORα), among others. The main features of each of these nuclear receptors have already been commented-upon previously.

Many published studies reinforce the assumption that APOA5 gene may be modulated by dietary components.

Firstly, several studies in humans have evaluated whether the relationship between dietary composition and plasma lipids are genetically determined by the APOA5 gene. Ordovas has reviewed all these studies (288) and highlighted that APOA5 is a major gene involved in TG metabolism and the effect is modulated by dietary factors, mainly

ISBN:978-84-691-9478-2/DL:T-19-2009

the fat composition. Some studies conducted in animal models also described a modulatory effect of dietary components on APOA5 gene expression, or on A-V protein levels. A recent (289) indicated that, in normal mice, oxidized fatty acids, which are also ligands for PPARα, decreased TG levels in plasma and, as expected, increased APOA5 gene expression and decreased APOC3 gene expression via a PPARα-independent mechanism. In Zucker rats, an animal model for hypertriglyceridemia and insulin resistance, the modulatory effect of ω-3 PUFA on APOA5 expression is mediated via SREBP-1 nuclear factor. However, in this case, mechanism proposed is that of co- or post-translational regulation, since liver and plasma apo A-V were not affected (290). The administration of squalene, an intermediate in the biosynthesis of cholesterol biosynthesis, for 10 weeks in APOE deficient mice has an impact on APOA5 gene expression and protein levels in a gender-dependent manner (291) i.e. in males, squalene significantly increases APOA5 gene expression in liver and decreases the hepatic fat content. The plasma TG levels were unaffected. In females, the hepatic expression of APOA5 was slightly increased, the fat content was unaffected, and there were lower levels of TG and the number of VLDL particles.

After we had observed the effect of a diet rich in saturated fat on hepatic expression of APOA5 (the down-regulatory effect reached the 75% in youngest mice) we measured the levels of circulating lipid, specially TG levels since, with a genetic background of 100% decrease in APOA5 gene expression (in KO-APOA5 mice), the TG levels have been described as being 4 times higher relative to wild type mice (149). Of note is that we found no variation in plasma TG levels among the different animal groups in relation to the type of diet. It is important, however, to highlight that the defect of APOE gene leads to a substantial intra-cellular accumulation of lipids, especially TG, due to impairment of VLDL-TG secretion (292) and, as such, any potential influence of the APOA5 gene on VLDL secretion system would be altered.

Another result of note was the significant increase in APOA5 gene expression in relation to age in all animals, irrespective of the type of diet received. An explanation for this observation could be that this animal model, when fed an atherogenic diet, develops inflammation, atherosclerosis, and severe steatosis with advancing age and which can account for hepatic lipid accumulation (293). As such, the increase in APOA5 expression with age would be a reflection of the age-related deterioration of the liver.

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We published data indicating that APOA5 expression in the liver of APOE deficient mice was correlated with inflammatory parameters; the relationship between APOA5 and inflammation having already been established. APOA5 gene has been studied in relation to acute-phase response (the cascade of reactions triggered by inflammation or infection) leading to increased TG levels and decreased HDLc levels in plasma. Apo A-V has been identified as a positive acute-phase protein which increases in plasma during inflammation induced by endotoxin-injected mice (167). A more recent study described a biphasic behavior with time of APOA5 gene expression in human hepatic HepG2 cells incubated with pro-inflammatory cytokines (tumor necrosis factor, TNFα; or interleukin, IL1β) and, as well, of plasma apo A-V and hepatic levels in mice injected with lipopolysaccharide; the result being an early decrease in mRNA and protein levels followed by a significant increase subsequently (168).

Secondly, we published data indicating that APOA5 expression in the liver of APOE deficient mice was correlated with the arteriosclerosis lesion area and, the correlation between APOA5 gene and the atherosclerotic lesion area was documented. This would make sense in the light of the data from association studies describing a significant effect on CHD incidence and/or prevalence (89, 188, 189, 211, 233, 236).

Thirdly, steatosis is the most common liver disease in industrialized countries. It is present in one third of the general population, and the incidence increases in patients with risk factors for cardiovascular disease and metabolic syndrome. The steatosis represents the hepatic consequence of relative over-nutrition and altered dietary composition, within the setting of sedentary behavior. During steatosis, there is up to a 10-fold accumulation of TG relative to normal. This could be due, among other explanations, to a greater fatty acid mobilization to the liver, or a diminished fatty acid oxidation, or increased intra-hepatic synthesis of fatty acids, TG or lipoproteins. The liver has the capacity to regenerate itself in response to cellular damage (294) and the energy necessary for this regeneration is obtained from lipids that have been accumulated during the first hours of the damage-limitation process; a period when APOA5 gene has been described, in the liver of a rat model of hepatic regeneration as being up-regulated more than 3-fold.

As such, we believe that the hepatic alteration undergone by APOE KO mice fed with atherogenic diet is responsible of the increase in the expression of APOA5, as has been postulated for the control of lipid uptake by the liver during the hepatic regeneration.

In conclusion, APOA5 gene expression is affected by dietary components in the APOE KO mouse and that it is correlated with inflammatory parameters and/or atherosclerosis lesions.

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With the APOA5 gene potentially implicated in steatosis development, we proceeded to analyze the hepatic APOA5 gene expression in 65 rats that received intra-peritoneal injections of 0.5 ml of carbon tetrachloride (CCl_4) diluted 1:1 (v/v) in olive oil (unpublished data). CCl_4 activates collagen synthesis, promotes fibrosis and steatosis in the liver via its effect on the morphology and/ or function of the lipoproteins which, as a result, fail to act as efficient vehicles for the transport of lipids from the liver into the circulation (295). We found that APOA5 expression following CCl_4 administration was 30% higher than in control rats ($p=0.019$). Of note is that we found that hepatic APOA5 expression levels were strongly and positively correlated with APOB expression levels, and inversely correlated with circulating cholesterol and TG levels in rats under CCl_4 exposure in a steatotic state. This suggests a role for APOA5 in the control of TRL release from the liver, as has been postulated in rats in the liver regeneration process (150).

Finally, to investigate further the function of apo A-V we designed studies for conduct in human intestinal tissue samples, and in human intestinal-cell culture.
The data published to-date regarding apo A-V levels in circulation suggested that its main function occurs predominantly at the intra-cellular level. Thus, in view of the results of the regulatory effect of dietary fat on APOA5 expression in the liver, our purpose was to test whether we could also find APOA5 expression in the other key organ for the lipoproteins synthesis, the intestine and which is also involved in the processing of dietary components.
We studied APOA5 gene, together with other genes involved in synthesis and secretion of TRL in intestine and liver (APOB and MTP) and in the metabolic regulation of TRL (APOC3). We used tissue samples from human intestine, and also *in vitro* culture of intestinal cells. We have been able to detect APOA5 gene expression and protein in human intestine. However, our results do not agree with published data (149, 150, 261) evaluating APOA5 in different animal tissues and/or different cell cultures. The conclusion for such data was that APOA5 is only expressed in the liver. This divergence of findings could be the result of methodological differences.

Initially, we analyzed the expression of the genes in the different intestinal tissue sections and found that each gene is differentially expressed along the extent of the intestine with APOA5 expressed mainly in duodenum and colon, and where significant and inverse correlation was observed with APOC3 gene expression. Apo A-V and apo C-III, two members of the apolipoprotein gene cluster on chromosome 11, are

ISBN:978-84-691-9478-2/DL:T-19-2009

considered to play antagonistic roles in the metabolism of TRL, at least in mice (296, 297, 149, 298), but our results suggest that this may also be valid in humans. Also, we found that APOA5 and C3 gene expression are inversely correlated in the *in vitro* model of human intestinal cells.

We further compared the expression levels measured in the intact human small intestine samples and samples obtained from human liver, and found that APOA5 gene is expressed about $3x10^4$-fold lower in intestine. However, our calculations indicate that the protein level in intestinal cells is about one third that detected in human hepatic cells. An explanation for these differences between hepatic and intestinal expression levels can be based on the postulated functions of apo A-V i.e. the regulation of TRL synthesis and/or secretion processes would be more important in the liver since this organ is key in the control of lipid homeostasis and, as well, it is the liver that is responsible for uptake of remnants from the circulation via the LDL-R family.

Secondly, we observed that APOA5 is expressed at low levels in human intestine. To obtain further evidence of the possible functionality of APOA5 gene in human small intestine we developed a series of experiments TC-7 cells from human colon carcinoma, a well-established intestinal cell model. The objective was to test whether the low expression levels are potentially affected by factors known to modulate TG levels, such as dietary fatty acids or a PPARα agonist. Hence, we tested different concentrations of long-chain fatty acids (known specific ligands for PPAR nuclear transcription factors to which APOA5 is responsive) on intestinal APOA5 expression in the CaCo2 / TC-7 cell model. We observed that monounsaturated and polyunsaturated fatty acids tended to decrease intestinal APOA5 expression while saturated fatty acids tended to increase the expression. This would imply that apo A-V would down-regulate intestinal lipid mobilization in a situation of high saturated fat intake.

However, this is does not accord with what we had reported from APOE-KO mice studies in which we observed that saturated fat significantly decreases hepatic APOA5 gene expression. One possible explanation for this discrepancy could be that the same stimuli produce different effects on gene expression and, as has been described for APOA1 gene (299), would depend on the tissue being explored. Also, we need to take into account that some difference could be expected when results in genetic modified mice are compared to those obtained in humans.

Since APOA5 gene was relatively high transcribed in colon, we proceeded to assess the modulatory effect of short-chain fatty acids (e.g. butyrate) on intestinal APOA5 expression. Over recent years the colon has come to be considered as a digestive organ, since bacterial fermentation takes place here in eliminating substrates that have

ISBN:978-84-691-9478-2/DL:T-19-2009

not been digested by human enzymes within the small intestine. These substrates include the dietary soluble fibers. Short-chain fatty acids are generated as a result of this fermentation in the colon, and these have important functions in relation to energy production, lipid and glucose metabolism. The three main short-chain fatty acids are acetate, propionate and butyrate. Acetate is the primary substrate for cholesterol synthesis but propionate which has hypolipidemic effects inhibiting cholesterol synthesis may also be generated (300). As such, the serum acetate : propionate ratio is positively related to serum cholesterol levels (301). With respect to lipid metabolism, butyrate has a lowering effect on triglycerides and cholesterol (302), it decreases hepatic and intestinal protein expression of apo B, MTP, apo A-I (303) and stimulates APOA4 gene expression. The result is an increased in the A-IV protein secretion and, as such, a modulation of reverse cholesterol transport (304).
Butyrate, in our cell culture experiments, induced a 4-fold increase in APOA5 expression and, as expected, APOB expression tended to decrease with increasing concentrations of butyrate. Without knowing the precise function of apo A-V in the intestine, we could speculate that the established hypolipidemic effects of butyrate are in accordance with the down-regulatory effect on APOA5 gene transcription.

Finally, as expected from results published in human hepatocytes (257), we found that APOA5 gene expression in intestinal TC-7 cells is also up-regulated by a PPARα agonist (Wy 14643). However APOB, APOC3 and MTP gene expression was decreased. The PPARα activated by the agonist hetero-dimerizes with other nuclear transcription factors such as retinoid X receptor (RXR), up-regulates LPL and APOA1, and down-regulates APOC3 (305) the result of which is a decrease in plasma TG concentrations i.e. a combined action of reduced triglyceride synthesis as well as increased catabolism.

In summary, our results demonstrate that APOA5 is not exclusively synthesized in the liver, but is to by found also in the intestine, where is expressed at low levels and is responsive to fatty acids and fibrates. Although the precise intestinal function of apo A-V is not clear, we can postulate that it may influence the absorption of lipids obtained from the diet and which are to be incorporated into chylomicrons in the form of TG. This expression occurs mainly in the first third of the intestine where lipid absorption takes place. Alternatively, the apo A-V may influence lipidation of chylomicrons or, perhaps, it is present in the intestine merely for incorporation into chylomicrons prior to entry into the circulation.

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patient-oriented and epidemiological research

Increased concentrations of circulating vitamin E in carriers of the apolipoprotein A5 gene −1131T>C variant and associations with plasma lipids and lipid peroxidation

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Abstract **The aim of this study was to investigate the effects of the apolipoprotein A5 (APOA5) 1131T>C gene variant on vitamin E status and lipid profile. The gene variant was determined in 297 healthy nonsmoking men aged 20–75 years and recruited in the VITAGE Project. Effects of the genotype on vitamin E in plasma, LDL, and buccal mucosa cells (BMC) as well as on cholesterol and triglyceride (TG) concentrations in plasma and apolipoprotein A-I (apoA-I), apoB, apoE, apoC-III, and plasma fatty acids were determined. Plasma malondialdehyde concentrations as a marker of in vivo lipid peroxidation were determined. C allele carriers showed significantly higher TG, VLDL, and LDL in plasma, higher cholesterol in VLDL and intermediate density lipoprotein, and higher plasma fatty acids. Plasma α-tocopherol (but not γ-tocopherol, LDL α- and γ-tocopherol, or BMC total vitamin E) was increased significantly in C allele carriers compared with homozygote T allele carriers ($P = 0.02$), but not after adjustment for cholesterol or TG. Plasma malondialdehyde concentrations did not differ between genotypes. In conclusion, higher plasma lipids in the TC+CC genotype are efficiently protected against lipid peroxidation by higher α-tocopherol concentrations. Lipid-standardized vitamin E should be used to reliably assess vitamin E status in genetic association studies.—** Sundl, I., M. Guardiola, G. Khoschsorur, R. Solà, J. C. Vallvé, G. Godàs, L. Masana, M. Maritschnegg, A. Meinitzer, N. Cardinault, J. M. Roob, E. Rock, B. M. Winklhofer-Roob, and J. Ribalta. **Increased concentrations of circulating vitamin E in carriers of the apolipoprotein A5 gene −1131T>C variant and associations with plasma lipids and lipid peroxidation.** ***J. Lipid Res.*** **2007.** 48: **2506–2513.**

Supplementary key words polymorphism • single nucleotide polymorphism • α-tocopherol • γ-tocopherol • triglycerides • cholesterol • plasma fatty acids • lipoproteins

Apolipoprotein A5 (APOA5) is located near the region of the APOA1/C3/A4 gene cluster on chromosome 11 involved in the regulation of triglyceride (TG) metabolism. The role that APOA5 plays in such regulation has been demonstrated extensively in genetically modified animal models (1–3) and in a large number of association studies (4–10). Mice overexpressing the APOA5 gene have 65% lower plasma TG, whereas the APOA5 knockout mouse develops ~4-fold higher TG concentrations (1). Association studies using different APOA5 markers have clearly shown that apolipoprotein A-V (apoA-V), despite its low plasma concentrations (11), is probably the strongest determinant of circulating TG concentrations (4–10). The most frequently analyzed variant is −1131T>C, and the C allele has been consistently associated with higher TG levels (4–10). The exact function of apoA-V is not known, although in vitro evidence shows that it may control plasma TG by downregulating hepatic VLDL synthesis and stimulating LPL activity (1, 12).

Circulating TG are transported within lipoproteins together with other lipophilic compounds such as vitamin E

Manuscript received 19 June 2007 and in revised form 26 July 2007 and in re-revised form 9 August 2007.

Published, JLR Papers in Press, August 10, 2007.
DOI 10.1194/jlr.M700285-JLR200

Abbreviations: apoA-V, apolipoprotein A-V; APOA5, apolipoprotein A5; BHT, butylated hydroxytoluene; BMC, buccal mucosa cell; BMI, body mass index; IDL, intermediate density lipoprotein; SFA, saturated fatty acid; TG, triglyceride.

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(13). Consequently, TG concentrations are closely related to vitamin E concentrations, resulting in higher plasma vitamin E concentrations in hyperlipidemic subjects compared with normolipidemic subjects when not corrected for plasma lipids (14).

Vitamin E is an essential fat-soluble nutrient comprising eight different isoforms, including α-, β-, γ-, and δ-tocopherols and their respective tocotrienols, which differ in both number and position of methyl groups on the chroman ring and in having a saturated (tocopherols) or unsaturated (tocotrienols) side chain. Together with dietary lipids, all of these vitamin E isoforms are absorbed equally from the gastrointestinal tract and secreted into chylomicrons. In the liver, the α-tocopherol transfer protein preferentially recognizes RRR-α-tocopherol for incorporation into lipoproteins, whereas the other vitamin E forms are excreted into bile and metabolized and excreted into urine (15, 16). Consequently, α-tocopherol, which also shows the highest biologic activity, is the major form of vitamin E in human plasma and tissue.

The best known function of vitamin E relates to its capacity to scavenge reactive oxygen species, thus acting as a chain-breaking antioxidant inhibiting lipid peroxidation. As an example, α-tocopherol inhibits LDL oxidation initiated by copper ions in an ex vivo model (17). Beside these antioxidant actions, vitamin E also possesses anti-inflammatory functions, such as inhibition of platelet adhesion, inhibition of monocyte proatherogenic activity, and improvement of endothelial functions (18).

Transfer of circulating vitamin E from lipoproteins into tissue is regulated by mechanisms that also control lipid metabolism. The enzyme LPL is able to transfer tocopherols during TG hydrolysis to extrahepatic tissues (19), whereas the phospholipid transfer protein exchanges α-tocopherol between different lipoprotein classes (20). Furthermore, vitamin E transported in LDL is delivered to cells via a receptor-mediated uptake of LDL (21). Consequently, lipid, lipoprotein, and vitamin E concentrations correlate strongly with each other, indicating that their metabolic pathways are tightly linked. Therefore, it is conceivable that environmental and genetic factors affecting lipid metabolism would affect vitamin E status.

The hypothesis of the present study was that the APOA5 genotype not only influences lipid metabolism but, as a result of the mechanisms described above, also alters vitamin E metabolism in humans. To test this hypothesis, we investigated the effects of the APOA5 variant −1131T>C on lipid status in a population of 297 healthy male nonsmoking subjects aged 20–75 years and whether these changes have repercussion on vitamin E status. A detailed lipoprotein profile, including total lipids and apolipoproteins plus the lipid and apolipoprotein contents of all lipoprotein fractions, was determined along with vitamin E concentrations in plasma, LDL, and buccal mucosa cells (BMC). In addition, we studied the effects of the APOA5 variant-associated differences in vitamin E and lipid status on malondialdehyde, a biomarker of in vivo lipid peroxidation, which has been used successfully in patients with increased plasma lipids (22).

METHODS

Study subjects

In this cross-sectional study, 299 healthy male nonsmoking subjects (0 cigarettes/day for >6 months), aged 20–75 years (stratified by age), were recruited in Clermont-Ferrand, France (n = 99), Graz, Austria (n = 100), and Reus, Spain (n = 100) as part of the European Commission-funded research and technology development project of the 5th Framework Program, specific research and technology development Program Quality of Life and Management of Living Resources, Key Action 1, Food, Nutrition, and Health, entitled Vitamin A, Vitamin E, and Carotenoid Status and Metabolism during Ageing: Functional and Nutritional Consequences, acronym VITAGE (Contract QLK1-CT-1999-00830) (23). After informative sessions, a trained medical doctor conducted a personal interview to obtain information on anthropometric measurements, personal history, lifestyle, use of medications, physical activity, smoking habits, and use of dietary supplements containing vitamins or trace elements. Exclusion criteria were familial hypercholesterolemia, chronic diseases (including diabetes, cancer, cardiac insufficiency, neurological diseases, inflammatory diseases and chronic diseases of the liver, lung, or thyroid, nonstable hypertension, dementia, and infectious diseases known to affect the immune system, such as human immunodeficiency virus and hepatitis C), vaccination during the past 2 months, alcoholism or drug addiction, competitive sports activities, and the consumption of special diets or dietary supplements in the past 3 months. The study protocol was approved by the Ethics Committees of the three recruiting centers, and written informed consent was obtained from all participants.

Sample collection

Collection of blood samples and preparation of plasma and buffy coats. After an overnight fast, venous blood was drawn into plastic tubes containing EDTA to obtain EDTA plasma (Sarstedt, Ltd., Nümbrecht, Germany), protected from light, and centrifuged immediately at 1,500 *g* at 8°C for 10 min. Plasma was separated and divided into aliquots. For LDL isolation, a 60% sucrose solution was added to plasma to obtain a final concentration of 0.6% (24). For genomic DNA isolation, buffy coat was collected from blood drawn on EDTA. All samples were stored at −80°C until determination of analytical variables.

Collection of BMC. After rinsing the mouth with drinking water, BMC were collected as described by Gilbert et al. (25). Briefly, study subjects were asked to brush the inside of their cheeks with a soft toothbrush 20 times on one side and rinse the mouth with 25 ml of isotonic table salt solution (0.9% sodium chloride) and then repeat the procedure on the other side. The two rinsing volumes were collected in a single tube and centrifuged at 1,400 *g* for 10 min at 4°C. The supernatant was discarded, and the cell pellet was washed with 15 ml of cold phosphate-buffered saline solution. After vortexing, the sample was centrifuged as above and the supernatant was removed. The cell pellet was resuspended in 1.2 ml of cold PBS, flushed with nitrogen, and stored at −80°C until determination of vitamin E.

Five day food records. For dietary assessment, subjects recorded all food items and drinks consumed during a 5 day period. On the basis of these food records, the intake of total fat, saturated fatty acids (SFA), MUFA, and PUFA was calculated. For calculation of nutrients, the REGAL food composition tables (26, 27) and the software package of the Austrian food composition table Ernährungswissenschaftliches Programm (dato Denkwerkzeuge, Vienna, 1997) were used.

Determination of plasma lipids

Determination of TG and cholesterol. TG and cholesterol concentrations in plasma and lipoprotein fractions were measured using enzymatic kits (F. Hoffmann-La Roche, Ltd.) adapted for a Cobas Mira centrifugal analyzer (F. Hoffmann-La Roche) with Precilip EL® and Precinorm® (F. Hoffmann-La Roche) as quality controls. Immunoturbidometry was used for measurement of the apolipoproteins using specific antiserum purchased from F. Hoffmann-La Roche (for apoA-I and apoB), Daiichi Chemicals (for apoE and apoC-III), and Incstar Corp. (for lipoprotein [a]).

Sequential preparative ultracentrifugation. Lipoproteins were separated by sequential preparative ultracentrifugation using a Kontron 45.6 fixed-angle rotor in a Centrikon 75 (Kontron Instruments). The following lipoprotein fractions were isolated: VLDL ($d < 1.006$ g/ml), intermediate density lipoprotein (IDL; $d = 1.006$–1.019 g/ml), and LDL ($d = 1.019$–1.063 g/ml). Total HDL cholesterol was measured subsequent to the precipitation of the apoB-containing lipoproteins with polyethylene glycol (Immuno AG).

Determination of plasma total fatty acids. The determination of the fatty acid content in plasma was based on an esterification procedure and a subsequent GC analysis of the fatty acid methyl esters as described by Sattler et al. (28). Briefly, 450 μl of EDTA plasma and 100 μl of internal standard (10 mg of heptadecanoic acid and 100 mg of butylated hydroxytoluene (BHT) in 10 ml of methanol) were added to a Teflon screw-capped tube, vortexed for 10 s, and then kept at −80°C for a minimum of 30 min. The deep-frozen suspension was freeze-dried on the lyophilisator (Virtis; Servo Laboratories, Graz, Austria) for ~15 h. Thereafter, 1 ml of boron trifluoride-methanol complex and 500 μl of toluene were added and vortexed for 20 s Transesterification was performed at 110°C for 90 min. After the transesterification step, 2 ml of purified water was added and the fatty acid methyl esters were extracted three times with *n*-hexane. The hexane extracts were dried in a Speed-Vac (Bachhofer; Servo Laboratories) at room temperature for 30 min, redissolved in 450 μl of dichloromethane, and subjected to GC analysis (Hewlett-Packard 5890 Series II; Agilent Technologies, Vienna, Austria). Separation of fatty acid methyl esters was achieved on a DB-23 column (Agilent Technologies) with a length of 30 m and a diameter of 0.250 mm. The mobile phase was a mixture of helium and hydrogen gas. The oven temperature at injection was 150°C and was increased to 222°C (3°C/min) and kept at this temperature for 3 min, then increased further to 238°C (3°C/min) and kept at this temperature for 3 min, and finally increased to 255°C (20°C/min). The areas under the GC peaks were quantified by integration, and the internal standard described above was used for calculation of the amounts of fatty acids. Nineteen plasma samples of the patients were processed along with a control sample from the plasma pool for long-term quality control. Coefficients of variation for the different fatty acids were between 0.38% and 8.3% within run and between 1.7% and 8.6% between run.

Determination of vitamin E

LDL isolation. LDL was isolated as described by Bergmann et al. (24). Briefly, 1.5264 g of solid potassium bromide was added to 4 ml of defrosted EDTA plasma, which had been mixed with sucrose as described above. Separation of LDL was achieved by single-step discontinuous gradient ultracentrifugation in a Beckman NVT65 rotor (Beckman Coulter, Servo Laboratories) at 60,000 rpm for 2 h at 10°C (29). Thereafter, the LDL band was isolated and filtered through a 0.20 μm sterile filter (Corning, Inc., Corning, NY) into an evacuated glass vial (BD Vacutainer; Belliver Industrial Estate, Plymouth, UK) and processed on the same day. LDL density was determined using an Anton Paar DMA 48 density meter (A. Paar, Ltd., Graz, Austria). The cholesterol content of LDL was determined using a kit from Roche Diagnostics Ltd. (F. Hoffmann-La Roche).

Determination of α- and γ-tocopherols in plasma and in LDL. The determination of tocopherols in plasma and in LDL was performed as described by Aebischer, Schierle, and Schuep (30). Briefly, EDTA plasma or isolated LDL was diluted with deionized distilled water and deproteinized with 400 μl of absolute ethanol. To extract lipophilic compounds, 800 μl of *n*-hexane/BHT (350 mg of BHT in 1,000 ml of *n*-hexane) was added and centrifuged, and the clear supernatant was transferred by a dispenser/dilutor system (Micro Lab 500B Dilutor; Hamilton, Martinsried, Germany) to an Eppendorf tube to be dried on a Speed-Vac (Savant, New York, NY). The residue was then redissolved in a mixture of methanol and 1,4-dioxane (1:1), diluted with acetonitrile, and injected into the HPLC system (Hewlett-Packard 1100A; Agilent). Separation was achieved on a reverse-phase column; the mobile phase was a mixture of acetonitrile, tetrahydrofuran, methanol, 1% ammonium acetate solution, and 10 mg of L(+)-ascorbate; the flow rate was 1.6 ml/min. Vitamin E was detected using a fluorescence detector (Jasco model FP-920; Biolab, Vienna, Austria) at extinction of 298 nm and emission of 328 nm (α- and γ-tocopherol). The areas under the HPLC peaks were quantified on an HP Chemstation (Hewlett-Packard 35900E; Agilent). The coefficients of variation within run were 1.26% for α-tocopherol and 0.80% for γ-tocopherol in plasma and 0.70% for α-tocopherol and 1.4% for γ-tocopherol in LDL. The coefficients of variation between day were 1.81% for α-tocopherol and 2.78% for γ-tocopherol in plasma and 2.54% for α-tocopherol and 2.98% for γ-tocopherol in LDL. Six plasma and LDL samples of the subjects were processed along with two control samples from the plasma and LDL pools, respectively, obtained from a number of healthy subjects to be used for long-term quality control along with a standard solution. The detection limit was 0.012 μmol/l for α- and γ-tocopherol. The tocopherol content of LDL was expressed as moles of tocopherol per mole of LDL.

Determination of total vitamin E in BMC. Vitamin E was extracted from a resuspension of BMC in PBS (1 ml) as described by Borel et al. (31). Briefly, after a short sonication (15 s; Labsonic U; B. Braun), α-tocopheryl acetate (Fluka, l'Isle d'Abeau, France) was added to samples as an internal standard. The proteins were precipitated with ethanol precipitation. Then, the vitamin E was extracted twice with hexane. The extract was evaporated to dryness under nitrogen, dissolved in methanol-dichloromethane (65:35, v/v), injected into a C18 column (5 μm, 250 mm × 4.6 mm; Nucléosil; Interchim, Montluçon, France), and assayed by reverse-phase HPLC (HPLC apparatus: Waters 996 UV-vis DAD; Waters SA, St-Quentin-en-Yvelines, France). Pure methanol, at a flow-rate of 2 ml/min, eluted α-tocopherol in 5.0 min and tocopheryl acetate in 6.3 min. The compounds were detected by ultraviolet light (292 nm), then quantified by internal and external calibration using daily-controlled standard solutions. Vitamin E concentrations were standardized for protein, as determined by Lowry et al. (32).

Determination of malondialdehyde in plasma

Plasma malondialdehyde concentrations were determined by HPLC as a biomarker of in vivo lipid peroxidation. The method described by Khoschsorur et al. (33) was used.

APOA5 genotyping

According to the nomenclature and methodology used by Pennacchio and colleagues (1), the single nucleotide polymorphism −1131T>C was used as the genetic marker. Genotyping was performed with primers AV-1 (5′-GATTGATTCAAGATGCATTTAGGAC-3′) and AV-2 (5′-CCCCAGGAACTGGAGCGAAATT-3′), which forced a *Mse*I (New England Biolabs, Beverly, MA) site for enzymatic restriction.

Statistical methods

Statistical analyses were carried out with SPSS version 14.0. The Chi-square test was used to test for Hardy-Weinberg equilibrium. Normal distribution of data was checked with the Kolmogorov-Smirnov test. Comparisons of age and body mass index (BMI) were made with Student's *t*-test. ANOVA was performed to compare the mean values of lipid, lipoprotein, apolipoprotein, and vitamin E data adjusted for age and BMI, because of well-known associations between plasma lipids, age, and BMI. Linear regression analysis was performed between plasma and LDL vitamin E concentrations. Log-transformation was performed when variables were not normally distributed. Results are expressed as means ± SD or box-and-whisker plots. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic data of the study population

We studied a total of 297 nonsmoking healthy males aged 20–75 years from France, Austria, and Spain. Genetic material was missing for two subjects; thus, two subjects had to be excluded from statistical analysis. There were no differences in mean age, age distribution, and BMI among countries (**Table 1**); therefore, all subjects were pooled for genetic analyses of lipid and vitamin concentrations.

Frequencies of the −1131T>C polymorphism in the APOA5 gene

Among the 297 subjects, 251 had the common genotype (T/T), 45 were heterozygote (T/C), and 1 subject was homozygote (C/C). For association analyses, all carriers of one or two copies of the C allele were pooled. The C allele was carried by 15.5% of the subjects, resulting in an allele frequency of 0.08, which was similar to that found in the Caucasian general population (6). The observed frequencies of the −1131T>C genotypes were not different from those predicted by the Hardy-Weinberg distribution. There were no differences in the allele frequencies between Spain, France, and Austria (Table 1).

Characteristics of the study subjects according to APOA5 genotype

There were no differences in age between homozygote carriers of the T allele (45.8 ± 15.1 years) and carriers of the C allele (48.0 ± 17.1 years). BMI did not differ between the TT (25.0 ± 2.66 kg/m^2) and the TC+CC (24.7 ± 2.65 kg/m^2) genotypes.

Effects of the APOA5 genotype on lipoprotein profile

Carriers of the C allele had 15.2% higher plasma TG concentrations ($P = 0.01$) attributable to increases in VLDL-TG ($P = 0.001$), whereas IDL-TG, LDL-TG, and HDL-TG did not differ significantly between the genotypes (**Table 2**). This was accompanied by a 10% increase in the lipoprotein lipase inhibitor apoC-III in the TC+CC genotype (17.0 ± 3.27 mg/dl) compared with the TT genotype (15.4 ± 3.30 mg/dl) ($P = 0.002$). Total cholesterol concentrations were not different between carriers and noncarriers of the C allele. However, carriers of the C allele showed significantly higher cholesterol concentrations in the VLDL ($P = 0.002$) and IDL ($P < 0.05$) fractions, whereas there were no differences in the LDL and HDL fractions (Table 2). Circulating apoB-containing lipoproteins were also 11% higher in carriers of the C allele (apoB-100; $P = 0.006$) as a result of an accumulation of VLDL and LDL particles, as assessed by their apoB content (VLDL, $P = 0.008$; LDL, $P = 0.03$). In contrast, the APOA5 genotype did not have an effect on HDL (Table 2). There was no difference between the TT and TC+CC genotypes in plasma lipoprotein [a] (23.5 ± 21.7 vs. 23.1 ± 20.0 mg/dl) and apoE (3.50 ± 0.872 vs. 3.48 ± 0.691 mg/dl).

Effects of dietary intake and of the APOA5 genotype on plasma fatty acids

There were no differences in total fat intake or in intake of SFA, MUFA, and PUFA between the genotypes. After dichotomizing dietary PUFA intake according to the study population mean (16.2 ± 6.95 g/day), there was no effect of high or low PUFA intake on TG concentrations, plasma VLDL, and VLDL-TG. Total plasma fatty acids were 8.6% higher in carriers of the C allele compared with homozygote carriers of the T allele (10.4 ± 2.31 vs. 11.3 ± 2.32 mmol/l) ($P = 0.007$). The distribution of fatty acids

TABLE 1. Characteristics of the study population

Characteristics	France (n = 97)	Austria (n = 100)	Spain (n = 100)	Total (n = 297)	P^a
Anthropometric variables					
Age (years)	46.6 ± 15.1	45.5 ± 15.1	46.3 ± 16.0	46.1 ± 15.4	NS
BMI (kg/m^2)	24.6 ± 2.58	25.2 ± 2.71	25.1 ± 2.66	25.0 ± 2.66	NS
Genotype					
TT (%)	84.5	86.0	83.0	84.5	NS^b
TC/CC (%)	15.5	14.0	17.0	15.5	NS^b
C allele frequency	0.08	0.07	0.09	0.08	NS^b

BMI, body mass index. Anthropometric variable values are shown as means ± SD.
[a] France versus Austria versus Spain.
[b] By Chi-square test.

TABLE 2. Lipoprotein profiles according to APOA5 genotype

	Cholesterol			TG			ApoB/ApoA-I Content		
Lipoprotein	TT (n = 251)	TC + CC (n = 46)	P^a	TT (n = 251)	TC + CC (n = 46)	P^a	TT (n = 251)	TC + CC (n = 46)	P^a
	mmol/l			*mmol/l*			*mg/dl*		
Plasma	4.82 ± 0.932	5.06 ± 0.878	NS[d]	1.05 ± 0.522	1.21 ± 0.582	0.01[d,e]	70.6 ± 17.2[b]	78.2 ± 17.4[b]	0.006[d]
							133.5 ± 18.9[c]	135.9 ± 17.89[c]	NS[d]
VLDL	0.298 ± 0.248	0.397 ± 0.290	0.002[d,e]	0.568 ± 0.419	0.751 ± 0.505	0.001[d,e]	3.16 ± 2.18[b]	4.15 ± 2.58[b]	0.008[d,e]
IDL	0.193 ± 0.106	0.224 ± 0.113	0.048[d,e]	0.093 ± 0.337	0.105 ± 0.442	NS[d,e]	2.73 ± 1.37[b]	3.11 ± 1.57[b]	NS[d,e]
LDL	2.68 ± 0.728	2.89 ± 0.652	NS[d]	0.237 ± 1.00	0.188 ± 0.048	NS[d,e]	54.4 ± 14.6[b]	59.7 ± 13.4[b]	0.03[d]
HDL	1.33 ± 0.308	1.33 ± 0.296	NS[d]	0.094 ± 0.031	0.103 ± 0.041	NS[d,e]	112.6 ± 17.4[c]	114.8 ± 16.6[c]	NS[d]

APOA5, apolipoprotein A5; IDL, intermediate density lipoprotein; TG, triglyceride. Values shown are means ± SD.
[a] ANOVA.
[b] ApoB.
[c] ApoA-I.
[d] *P* value standardized for BMI and age.
[e] On log-transformed data.

among SFA, MUFA, and n-3 or n-6 PUFA expressed as mol% did not differ between the genotypes.

In vivo lipid peroxidation according to APOA5 genotype

Plasma malondialdehyde concentrations did not show a significant difference between the TT and TC+CC genotypes, either when the concentrations (TT, 0.68 ± 0.34 μmol/l vs. TC+CC, 0.75 ± 0.38 μmol/l) or when the ratios of malondialdehyde to cholesterol (TT, 0.144 ± 0.074 μmol/mmol vs. TC+CC, 0.155 ± 0.086 μmol/mmol) were used.

Effects of the APOA5 gene on vitamin E status

Carriers of the C allele had significantly higher (9.1%) plasma α-tocopherol concentrations compared with noncarriers ($P = 0.02$) (**Fig. 1**). Because there was a significant relation between plasma α-tocopherol concentrations and TG concentrations ($r = 0.50$, $P < 0.001$) and cholesterol concentrations ($r = 0.75$, $P < 0.001$), standardization was performed. The difference in α-tocopherol concentrations between the genotypes was not statistically significant when standardized for TG and cholesterol (both separately and in combination), nor when measured in the LDL fraction. γ-Tocopherol concentrations as well as total vitamin E concentrations measured in BMC did not differ significantly between carriers and noncarriers of the C allele (**Table 3**). There was a close relation between plasma and LDL α- and γ-tocopherol concentrations in both genotypes (α-tocopherol, $r = 0.45$, $P < 0.001$ in T/T, $r = 0.64$, $P < 0.001$ in C/T and CC; γ-tocopherol, $r = 0.93$, $P < 0.001$ in T/T, $r = 0.94$, $P < 0.001$ in C/T and CC).

DISCUSSION

The hypothesis of this study was that, through its action on lipid metabolism, the APOA5 gene would have an effect on the metabolism and distribution of the fat-soluble vitamin E and thus could alter the status of this most potent lipophilic antioxidant. In the study subjects (i.e., healthy male nonsmoking volunteers), the APOA5 variant −1131T>C had a significant impact on plasma vitamin E concentrations, such that the carriers of the C allele exhibited high α-tocopherol but not γ-tocopherol concentrations in association with increased TG concentrations compared with carriers of the T allele. TG concentrations are well known to be a major determinant of circulating vitamin E concentrations (14); therefore, standardization

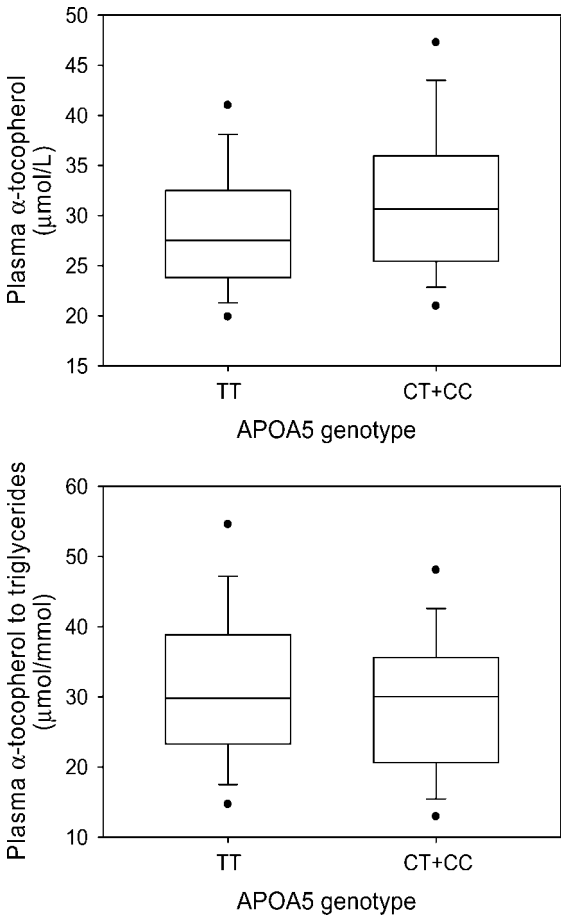

Fig. 1. Differences in plasma α-tocopherol concentrations ($P = 0.02$; upper panel) and α-tocopherol standardized for triglyceride concentrations (not significant; lower panel) between apolipoprotein A5 (APOA5) genotypes. Results are expressed as box-and-whisker plots.

TABLE 3. Vitamin E profile according to APOA5 genotype

Vitamin E Status	APOA5 Genotype		
	TT (n = 251)	TC + CC (n = 46)	P^a
Plasma α-tocopherol (μmol/l)	28.7 ± 6.34	31.3 ± 7.43	0.02[b,c]
Adjusted for cholesterol			NS[b,c]
Adjusted for TG			NS[b,c]
Adjusted for cholesterol and TG			NS[b,c]
Adjusted for apoB			NS[b,c]
Plasma α-tocopherol to cholesterol (μmol/mmol)	5.96 ± 0.907	6.15 ± 1.03	NS[b,c]
Plasma γ-tocopherol (μmol/l)	1.38 ± 0.835	1.46 ± 0.689	NS[b,c]
Plasma γ-tocopherol to cholesterol (μmol/mmol)	0.285 ± 0.154	0.290 ± 0.126	NS[b,c]
LDL α-tocopherol (μmol/l)	8.28 ± 1.10	8.51 ± 1.25	NS[b]
LDL γ-tocopherol (μmol/l)	0.329 ± 0.180	0.329 ± 0.138	NS[b,c]
Buccal mucosa cell total vitamin E (ng/mg protein)	119.0 ± 95.8	110.0 ± 55.6	NS[b,c]

Values shown are means ± SD.
[a] ANOVA.
[b] *P* value standardized for BMI and age.
[c] On log-transformed data.

for TG concentrations was performed. After standardization, the effect of the genotype disappeared, indicating that increased vitamin E concentrations are a function of increased TG concentrations. This relationship is further supported by the highly significant linear regression of α-tocopherol concentrations on TG concentrations in the present study.

APOA5 and vitamin E

The reason for this differential effect of the −1131T>C variant of the APOA5 gene on plasma α-tocopherol compared with γ-tocopherol concentrations may relate to the fact that, although intestinal absorption of the different vitamin E isoforms is similar, α-tocopherol transfer protein in the liver preferentially binds the α-tocopherol, which is then incorporated into nascent VLDL particles (15, 16). Although this metabolic pathway is responsible for the enrichment of α-tocopherol in lipoproteins and, consequently, also in tissues, γ-tocopherol is either excreted via bile or metabolized in the liver to be excreted as the water-soluble γ-carboxyethyl-hydroxychroman in the urine (15, 16). Because in vitro data suggest that apoA-V may influence hepatic VLDL synthesis (34), the incorporation of α-tocopherol into VLDL could be modulated by the −1131T>C genotype. This results not only in higher TG concentrations in the carriers of the C allele but also in higher α-tocopherol concentrations. In contrast, because of the lack of incorporation of γ-tocopherol into nascent VLDL, γ-tocopherol concentrations are not affected by the genotype. This could explain the absence of genotype-related differences in both plasma and LDL γ-tocopherol concentrations observed in the present study.

The extrahepatic tissue uptake and distribution of vitamin E is directed mainly by LPL (19). Because apoA-V stimulates LPL activity (2, 35), the APOA5 genotype could also influence the distribution of vitamin E between plasma and tissues. Our results indicate that increased α-tocopherol concentrations are entirely attributable to increased plasma lipid concentrations. Given that there was no difference in LDL-TG content between the genotypes, it is not surprising that there was also no difference in LDL α-tocopherol concentrations. Because micronutrient concentrations in BMC have been suggested to reflect vitamin E status better than plasma concentrations (36), we determined vitamin E in BMC. There were no differences between the APOA5 genotypes in BMC vitamin E, in agreement with LDL vitamin E concentrations and vitamin E plasma concentrations standardized for lipid concentrations.

APOA5 and lipids and lipoproteins

The crucial role of apoA-V in TG metabolism has been demonstrated in several transgenic and knockout animal models (1–3) as well as in epidemiological studies investigating single nucleotide polymorphisms at the human APOA5 locus (5, 9, 10). In the present study, we aimed to study its effect on a population of subjects of different ages specifically recruited to qualify as healthy and with a complete lipid and lipoprotein profile. Carriers of the C allele had increased TG concentrations compared with wild-type subjects, which were mainly attributable to increases of TG in the VLDL fraction. Carriers of the C allele also had higher VLDL and IDL cholesterol and higher apoB concentrations, indicating a significantly higher number of apoB-containing lipoproteins. Similar observations have been made previously (2, 37). However, it remains to be determined whether increased synthesis, decreased clearance of TG-rich lipoproteins, or both are the underlying mechanisms of the effects of apoA-V on lipid status. Our results support both possibilities, because carriers of the C allele had increased plasma concentrations of free fatty acids and apoC-III. Although increased concentrations of free fatty acids are known to induce hepatic synthesis of VLDL, increased concentrations of apoC-III can impair the ability to hydrolyze TG as well as the removal of lipoprotein remnants. This could explain all of the observed features associated with the −1131T>C gene variant, namely, increased VLDL, increased TG, and increased IDL (VLDL remnants).

Increased plasma fatty acid concentrations did not show a specific pattern of increased fatty acids. Recently, a modulating effect of dietary n-6 PUFA on plasma lipids in carriers of the C allele was shown (38). However, in the present study, such an effect was not observed.

This study was not designed to test the hypothesis that differences in vitamin E status attributable to the APOA5 variant −1131T>C would affect biomarkers of oxidative stress and inflammation. Only healthy volunteers were enrolled who had passed strict inclusion/exclusion criteria, such that they did not have increased markers of inflammation such as C-reactive protein, leukocyte count, or bands. At the same time, they had to be nonsmokers, with BMI < 30 kg/m^2, and free of acute or chronic diseases, making an increased oxidative stress status very unlikely. They were also in a fasted state, such that postprandial lipid peroxidation would not be investigated. Furthermore, plasma α-tocopherol standardized for lipids [i.e., α-tocopherol to cholesterol of 5.96 ± 0.91 μmol/l (TT) compared with 6.15 ± 1.03 μmol/l (TC+CC)] was not associated with significant differences in lipid peroxidation, because higher lipids in the TC+CC genotype are associated with higher α-tocopherol concentrations, resulting in comparable protection against lipid peroxidation.

In summary, the data presented here indicate that the APOA5 gene significantly alters the lipoprotein profile even in healthy subjects. Such an effect is accompanied by increased circulating vitamin E concentrations as a result of increased TG concentrations, whereas vitamin E metabolism does not seem to be affected. From a health perspective, the association of increased plasma lipids with an increase in α-tocopherol concentrations is highly relevant for ensuring efficient protection against lipid peroxidation. These results should be taken into account when interpreting plasma vitamin E concentrations in humans. Given the fact that the −1131T>C variant affects lipid status and, as a consequence, also alters plasma vitamin E concentrations, this study further supports the use of vitamin E standardized for lipids to reliably assess vitamin E status, particularly in genetic association studies.

This study was carried out with financial support from the Commission of the European Communities, specific research and technology development program Quality of Life and Management of Living Resources, QLK1-CT-1999-00830, acronym VITAGE. Additional support by the government of Austria (Bundesministerium für Bildung, Wissenschaft und Kultur) and DSM Nutritional Products, Switzerland, is gratefully acknowledged. This study was also supported by Grant SAF 2002-02781 of the Spanish Ministry of Education and Science and the Thematic Network ISCIII-RETIC RD06. I.S. was supported by a KUWI Stipend (Kurzfristige Wissenschaftliche Arbeiten für Studierende und Graduierte) of the Austrian Government (Bundesministerium für Bildung, Wissenschaft und Kultur) and a Research Stipend of the Karl-Franzens University. M.G. is the recipient of a predoctoral fellowship from the Spanish Ministry of Education and Science (BES-2003-1090). This publication has been printed with support of the Karl-Franzens University of Graz.

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ISBN:978-84-691-9478-2/DL:T-19-2009
Clin Chem Lab Med 2008;46(4):453–457 © 2008 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2008.110

The apolipoprotein A5 gene –1131*T*→*C* polymorphism affects vitamin E plasma concentrations in type 2 diabetic patients

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Abstract

Background: Variations of the apolipoprotein A5 (*APOA5*) gene are strongly associated with hypertriglyceridemia. Vitamin E is transported in triglyceride (TG)-rich lipoproteins and therefore could also be modulated by apoAV. Patients with type 2 diabetes have a tendency towards high TG values and increased oxidative stress.
Methods: We examined the impact of genetic *APOA5* variation (–1131*T*→*C*) on vitamin E and oxidative status in 169 non-smoker type 2 diabetic patients. Plasma samples were analyzed for lipids, lipoproteins, vitamin E, oxidized low-density lipoprotein (oxLDL), lipoperoxides, autoantibodies against oxLDL and diene formation of LDL.
Results: Vitamin E concentrations were higher in *TC* carriers compared with *TT* carriers (45.48±8.20 μmol/L vs. 40.32±10.47 μmol/L; p=0.02). The prevalence of the *TC* genotype was 2.6-fold higher among individuals with high vitamin E concentrations (p=0.02). The *APOA5* polymorphism did not determine any differences in oxidative status. Fasting TG concentration was a significant 21% higher in carriers of the *TC* genotype (p=0.04) due to higher TG concentrations in very-low-density lipoprotein (VLDL) and high-density lipoprotein.
Conclusions: The *APOA5*–1131*T*→*C* polymorphism is associated with both higher vitamin E concentrations and higher VLDL-TGs in diabetic patients.
Clin Chem Lab Med 2008;46:453–7.

Keywords: *APOA5*; oxidation; triglycerides; type 2 diabetes; vitamin E.

Introduction

Lipid oxidation is a crucial mechanism in the pathogenesis of diabetes and atherosclerosis (1). Diabetes is associated with increased oxidative stress that contributes to an accelerated predisposition to vascular damage. It has been observed by us and others that oxidative stress plays a casual role in macro- and microvascular lesions in both experimental and clinical diabetes (2, 3). Although the role of oxidation has been well established as a pathogenic mechanism for diabetes complications, the effectiveness of antioxidants is rather controversial. While in epidemiological studies those populations with high concentrations of natural antioxidants, such as vitamin E, have less risk for cardiovascular and metabolic problems (4), the administration of antioxidant supplements as vitamins C and E do not induce a reduction in neither diabetes predisposition nor cardiovascular complications (5). This is probably due to the differential effect of naturally acquired antioxidants vs. pharmacological administration (6). Among other effects, oxidative stress alters lipoproteins. The presence of low-density lipoprotein (LDL) oxidation markers in plasma is associated with vascular disease (7). Oxidized lipoproteins are incorporated into macrophages leading to foam cell formation and artery wall inflammation characterized by inflammatory cell recruitment and arteriosclerosis plaque progression (8, 9). The oxidative status results from the imbalance between pro-oxidant and antioxidant mechanisms. Because one of the main in vivo antioxidant molecules is vitamin E, it seems important to go deeply into its metabolic determinants.

Vitamin E is a generic name applied to a group of tocopherol and tocotrienol molecules that have antioxidant properties. It travels with lipoproteins and is therefore a good candidate to modulate lipoprotein oxidative status (10). It is obtained from different foods, absorbed by the enterocyte and then incorporated into chylomicrons to reach the blood stream. Chylomicrons are then catabolized by lipoprotein lipase (LPL); during this process, some vitamin E content is transferred to extra-hepatic tissues (11). Remnants are taken up by the liver where vitamin E molecules are made available to be incorporated into very-low-density lipoprotein (VLDL). The presence of a specific protein, α-tocopherol-transfer protein, plays an important role in selectively delivering α-tocopherol molecules to triglyceride (TG)-rich lipoprotein (TRL) in the hepatocyte (12). Vitamin E again reaches the blood stream inside VLDL; when the lipoprotein interacts with LPL, it is distributed to extra-hepatic tissues.

In the last few years, the apolipoprotein A5 (*APOA5*) gene has been strongly associated with plasma TG concentrations (13). Knockout animals for this gene have very high concentrations of TG, while transgenic mice show a low plasma concentration (14). In diabetic patients, carriers of *APOA5*–1131*T*→*C* gene variants are associated with low apoAV plasma concentrations (15) and hypertriglyceridemia (16, 17). It has been suggested that part of its effect could be

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Received September 20, 2007; accepted January 2, 2008; previously published online February 27, 2008

related to LPL activation (18), although it has also been suggested that the main effect of this apoprotein takes place intracellularly in the liver during VLDL formation (19).

Because vitamin E is a liposoluble molecule with a metabolism closely related to TRL, and apoAV is a key regulator of TG metabolism, it is interesting to study if genetic polymorphisms associated with hypertriglyceridemia could be associated with variations in vitamin E plasma concentration leading to changes in the oxidative status. These can have more importance in diabetic patients, because diabetes is a prooxidant condition and oxidative stress has been causally related to both micro- and macrovascular disease. Secondly, type 2 diabetes is associated with hypertriglyceridemia, and those genetic factors influencing VLDL metabolism can contribute to atherogenic dyslipidemia [high TG, low high-density lipoprotein (HDL) and accumulation of small and dense LDL] observed in this group of patients (20). In this paper we have studied, for the first time, the impact of the –1131$T \rightarrow C$ polymorphism of *APOA5* on vitamin E plasma concentrations in a group of non-smoker type 2 diabetic patients and its impact on lipid peroxidation status.

Materials and methods

Patients

The study group comprised 169 non-smoker type 2 diabetic patients (36–79 years) diagnosed in accordance with the American Diabetes Association (21). They were recruited in the Hospital Universitari Sant Joan de Reus. A complete medical history and clinical examination, including anthropometrics, blood pressure and retinal examination, were carried out. The presence of atherosclerosis was assessed by clinical history of coronary heart disease, stroke or peripheral vascular disease and/or when subjects presented at least one significant arteriosclerotic plaque ($>40\%$ stenosis) assessed by carotid and femoral eco-Doppler and/or when subjects had an ankle brachial index ≤ 0.9 or ≥ 1.3. Patients with type 1 diabetes mellitus, secondary diabetes mellitus, morbid obesity [body mass index (BMI) >40 kg/m^2], familial hypercholesterolemia, diabetic retinopathy and/or nephropathy, malignancy, liver disorders, acute or chronic inflammation and smokers (one cigarette in the last year) were not included. Informed consent was obtained from all patients and the protocol was approved by the Ethical Committee of Sant Joan University Hospital, Reus, Spain.

Chemical analyses

After a 12-h overnight fasting period, 20 mL of venous blood was taken into EDTA tubes and centrifuged immediately for 15 min at 4°C for $1500 \times g$. Plasma lipids, apolipoproteins, glucose and general biochemical parameters were measured using standard assays adapted for the Cobas-Mira autoanalyzer (Roche Diagnostic, Basel, Switzerland) and HbA_{1c} by high performance liquid chromatography (HPLC).

Lipoproteins were subfractionated by sequential preparative ultracentrifugation in a Kontron 45.6 rotor (Kontron Instruments, Milan, Italy) as described previously (22). Consecutive runs were carried out to obtain VLDL, intermediate density lipoprotein (IDL), LDL and HDL.

We used the solid phase two-site enzyme immunoassay Mercodia Oxidized LDL ELISA (Mercodia, Uppsala, Sweden) for the quantitative measurement of oxidized low-density lipoproteins (oxLDLs) in plasma. Results were calculated using the computerized data reduction of absorbance for the standards vs. the concentration using cubic spline regression; all results were expressed as U/L. The performance characteristics for this assay were 6.3% coefficient of variation (CV) intraassay and 4.7% CV interassay.

For the quantitative determination of anti-oxLDL antibodies (oxLDL-Ab) in plasma, we used an enzyme immunoassay with IMTEC-oxLDL-antibodies (IMTEC Immunodiagnostika GmbH, Berlin, Germany). This test permits the synchronous determination of immunoglobulin (Ig)G and IgM anti-oxLDL autoantibodies after a subtraction of anti-native-LDL autoantibodies. Results were calculated using the computerized data reduction of absorbance for the standards vs. the concentration using lineal regression and were expressed as U/mL. The performance characteristics of this assay were 7.8% CV intraassay and 8.6% CV interassay.

We used the colorimetric assay OxyStat (Biomedica, Vienna, Austria) for the quantitative determination of peroxides in plasma (LPO). Results are expressed in μmol/L. The performance characteristics for this assay were 3.1% CV intraassay and 5.1% CV interassay.

After extensive dialysis against phosphate-buffered saline at 4°C, the conjugated diene formation in LDL (50 μg/mL) was analyzed by monitoring the change at 234 nm at 30°C in an Uvikon spectrophotometer 922 (Kontron Instruments) in the presence of 6 μmol/L of cupric chloride dehydrate. Absorbance was recorded every 5 min for at least 5 h (23). Conjugated diene formation was measured in the LDL fraction from each subject.

Vitamin E measurement

Vitamin E (α-tocopherol) content was determined by HPLC (Hewlett-Packard, 1050 series, Waldbronn, Germany) as described by Catignani and Bieri (24) using tocopherol acetate as an internal standard (50 μg/mL). The column was Spherisorb ODS2, 125×4 mm, 5 μm (Teknokroma, Barcelona, Spain) and the mobile phase was methanol (100%). The HPLC instrument was equipped with a UV-visible detector and absorbance was recorded at 292 nm. The threshold value for high vitamin E values was chosen according to local population studies (>38.8 μmol/L for males and >41.6 μmol/L for females) (25).

DNA extraction and genotyping

Genomic DNA was extracted from whole blood using a commercial kit (Qiagen, Barcelona, Spain). The *APOA5*–1131$T \rightarrow C$ polymorphism was determined by PCR amplification and enzymatic restriction with *Mse*I (14).

Statistical analysis

Analysis was performed using SPSS (version 13.0, SPSS Inc., Chicago, IL, USA). All data are presented as the mean (SD). A comparison of variables between groups was performed using one-way analysis of variance (ANOVA). Categories were compared between groups using the χ^2-test. Genotype frequencies were estimated by the χ^2-test. In all cases, a p-value <0.05 was considered statistically significant.

Results

Among the 169 patients, 144 had the *TT* genotype and 25 were *TC* heterozygotes. The *C* allelic frequency

was 0.07, which was not different from that predicted by the Hardy-Weinberg distribution.

Table 1 shows the clinical and biochemical characteristics of the diabetic patients, including risk factors and therapy according to the –1131*T*→*C* polymorphism of *APOA5*. There were no significant differences between the two groups in terms of BMI, blood pressure, glucose or HbA_{1c}. All patients were non-smokers. Plasma concentrations of TG, apolipoprotein A-1 (apoAI) and apolipoprotein C-III (apoCIII) were significantly higher in *TC* carriers compared with *TT* carriers ($p < 0.05$). These results were confirmed when they were adjusted for hypolipidemic treatment.

Vitamin E concentrations were higher in *TC* carriers compared with *TT* carriers (45.48±8.20 μmol/L vs. 40.32±10.47 μmol/L, respectively; $p = 0.02$) (Table 2). The ratio of vitamin E to total cholesterol was significantly higher in the *TC* carriers compared to *TT* carriers (9.59±1.85 mmol/mol vs. 8.67±1.91 mmol/mol, respectively; $p = 0.027$). These results were confirmed when they were adjusted for age, gender and BMI. Figure 1 shows that the prevalence of the *TC* genotype was higher among those individuals with high vitamin E concentrations compared to the low vitamin E group (72% vs. 28%, respectively; $p = 0.022$), while the *TT* genotype was similarly distributed. There were no differences in lipid peroxidation markers (oxLDL, oxLDL-Ab, LPO, dienes) between *TT* homozygotes and *TC* carriers (Table 2).

Figure 2 shows the TG distribution in plasma lipoproteins for *APOA5*–1131*T*→*C* genotypes. As expected, the fasting plasma TG concentration was 21% higher in carriers of the *TC* genotype of *APOA5* ($p = 0.04$). This difference was due to higher TG in VLDL (0.96±0.78 mmol/L for *TT* carriers and 1.33±1.11 mmol/L for *TC* carriers; $p = 0.043$) and HDL fractions (0.14±0.05 mmol/L for *TT* carriers and 0.17±0.03 mmol/L for *TC* carriers; $p = 0.017$).

Discussion

Type 2 diabetic patients with the *APOA5*–1131*T*→*C* polymorphism had increased vitamin E concentrations. This increase in vitamin E was associated with higher plasma TGs and VLDL and HDL TG concentrations in this group of diabetic patients. The association between this polymorphism and hyper-

Table 1 Clinical, metabolic and lipid parameters according to the –1131*T*→*C* polymorphism of *APOA5* in type 2 diabetic patients.

	TT (n=144)	*TC* (n=25)	p-Value
Age, years	63.10±8.98	60.92±11.57	NS
Gender, males (%)	68 (47%)	12 (48%)	NS
Diabetes duration, years	14.52±7.77	12.36±6.95	NS
BMI, kg/m^2	30.12±4.28	30.70±4.52	NS
Glucose, mmol/L	8.88±2.99	9.42±3.72	NS
HbA_{1c}, %	7.02±1.09	6.87±1.31	NS
Triglycerides, mmol/L	1.69±0.94	2.14±1.29	0.043
Cholesterol, mmol/L	4.68±0.79	4.79±0.63	NS
LDL cholesterol, mmol/L	2.83±0.69	2.68±0.76	NS
HDL cholesterol, mmol/L	1.09±0.28	1.15±0.31	NS
Apolipoprotein A-I, g/L	1.37±0.20	1.48±0.22	0.017
Apolipoprotein B, g/L	0.84±0.16	0.85±0.11	NS
Apolipoprotein C-III, g/L	0.17±0.04	0.19±0.05	0.054
Apolipoprotein E, g/L	0.04±0.01	0.04±0.01	NS
Lipoprotein (a), g/L	0.31±0.32	0.31±0.31	NS
Systolic blood pressure, mm Hg	140.57±18.62	137.83±17.70	NS
Diastolic blood pressure, mm Hg	79.57±11.10	78.08±8.02	NS
Atherosclerosis, yes (%)	63 (44%)	10 (42%)	NS
Hypolipidemic treatment, yes (%)	76 (53%)	12 (48%)	NS

Values are given as mean±SD, number of patients, or percentage of patients. p-Values were calculated by one-way ANOVA or the χ^2-test for continuous and categorical variables, respectively.

Table 2 Vitamin E concentrations and lipid peroxidation parameters according to the –1131*T*→*C* polymorphism of *APOA5* in type 2 diabetic patients.

	TT (n=144)	*TC* (n=25)	p-Value
Vitamin E, μmol/L	40.32±10.47	45.48±8.20	0.020
Vitamin E/cholesterol, mmol/mol	8.67±1.91	9.59±1.85	0.027
Vitamin E/triglycerides, mmol/mol	27.99±11.69	26.33±12.85	0.519
LDL dienes formation			
Lag phase, min	82.70±20.98	78.30±21.54	NS
Maximal rate, mol/min	9.15±2.26	9.57±2.19	NS
Maximum diene formation, mol/mol	648.24±87.98	644.91±77.05	NS
Lipoperoxides, μmol/L	340.78±167.35	307.76±182.27	NS
OxLDL, U/L	68.86±22.13	67.54±16.19	NS
OxLDL-Ab, U/mL	21.83±9.03	22.00±9.18	NS

Values are given as mean±SD. p-Values were calculated by one-way ANOVA.

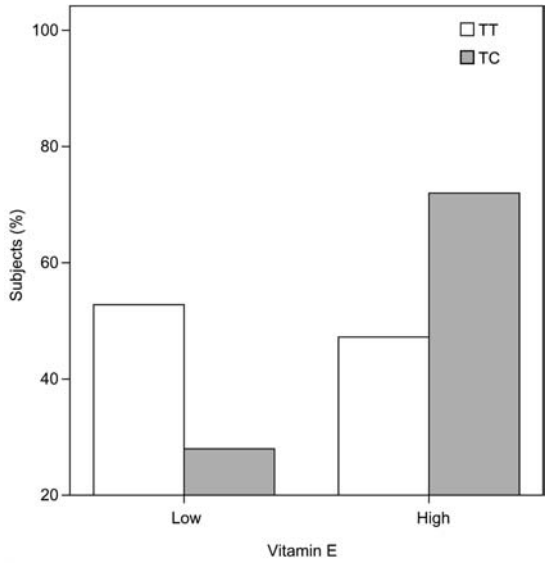

Figure 1 Distribution of different *APOA5* genotypes in type 2 diabetes according to high and low vitamin E concentrations. The threshold value for vitamin E is 38.8 μmol/L for males and 41.6 μmol/L for females (25). $\chi^2 = 5.233$, $p = 0.022$.

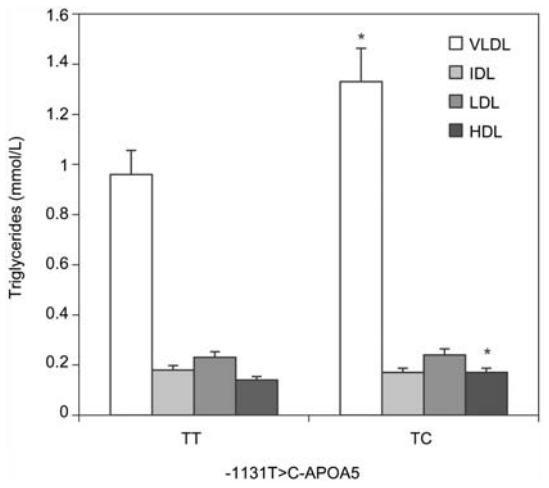

Figure 2 Triglyceride distribution in plasma lipoproteins for *APOA5*–1131*T*→*C* genotypes (*TT* = 144, *TC* = 25). Asterisks represent significant differences between genotypes. p-Values were calculated by one-way ANOVA.

triglyceridemia confirms other results, showing that the *APOA5* gene is strongly implicated in the regulation of TG metabolism (26–28). This effect is especially important in this group, because diabetes is a metabolic abnormality and patients with this condition are prone to hypertriglyceridemia.

In our study, we showed an increase in the apoCIII and apoAI levels in *C* carriers. The increase in the apoCIII levels correlates with the increase in TG levels in normolipidemic subjects, which suggests that it is more a consequence than a cause of the hypertriglyceridemia. These data would be in accordance with a putative coordinated regulation of the *A1-C3-A4-A5* gene cluster. Although around 50% of subjects in the study were treated with lipid-lowering drugs, the impact of this treatment on lipids was not affected.

Vitamin E is considered to be the main lipid antioxidant molecule. ApoAV seems to play a role in hepatic VLDL production (19), hence it could modulate the amount of vitamin E incorporated into TRL and thus rendered to the circulation. In this study, we observed that the *APOA5*–1131*T*→*C* genetic variant that is strongly associated with higher concentrations of TG is also associated with higher concentrations of vitamin E. The group of patients with this polymorphism had higher vitamin E concentrations. Furthermore, among patients with higher vitamin E concentrations the prevalence of the *APOA5*–1131*T*→*C* polymorphism was also higher. Interesting enough, the vitamin E to cholesterol ratio was increased in the group of patients wearing the rare allele; this is probably important so as to explain the lipoprotein antioxidant status.

It is already known that vitamin E increases with TG levels. It has been recently confirmed in relation to the *APOA5* gene (29).

An increase in circulating TG and small dense LDL could mismatch the molecular ratio necessary for keeping the antioxidant capacity of vitamin E. In this study, we show that this is not the case. The same genetic conditioning that leads to hypertriglyceridemia induces a higher presence of vitamin E. In our study, the higher vitamin E concentration was not associated with improved antioxidant parameters, such as peroxide concentration, diene formation, oxLDL or anti-oxLDL antibodies. However, Jang et al. (30) found that the presence of the –1131*C* allele had significant effects on oxidative parameters in a group of healthy Korean men. These different findings might be due to the type of population, presence of smokers and mainly the marked differences in the frequency of this particular polymorphism (0.28 in Koreans vs. 0.07 in Spanish). Moreover, vitamin E functions seem to be beyond anti-oxidation; recently, it has been shown that vitamin E acts as an intracellular trafficking signal and genetic regulator. In addition, it contributes to anti-inflammatory and immune mechanisms. The homeostasis of vitamin E seems to be important in maintaining redox status, as well as intracellular and intercellular connections (31). The molecular function of apoAV is not known, therefore we cannot speculate about the effect of *APOA5* on vitamin E metabolism. The plasma concentrations of *APOA5* appear to be very low and have no correlation with lipid metabolism. Therefore, it is considered that the main action of *APOA5* should be at the tissue level, especially in the liver, probably playing a role in the VLDL assembly process (19). The liver is where vitamin E should be incorporated into TRL and is probably influenced by *APOA5*.

In summary, those genetic situations prone to lower *APOA5* concentrations (32) will increase a tendency towards hypertriglyceridemia, but also counterbalance higher vitamin E concentrations, thus modulating the deleterious pro-oxidant effect of TG-enriched lipoproteins. This is especially important in oxidation prone diseases, such as type 2 diabetes. An enhanced knowledge of the genetic conditioning of vitamin E

metabolism will help us to better understand the molecular basis of these mechanisms.

Acknowledgements

This study was supported by a grant from the Instituto de Salud Carlos III (FIS 01/0398) and the CIBERDEM. Montse Guardiola is a recipient of a predoctoral fellowship at the Spanish Ministry of Education and Science (BES-2003-1090).

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UNIVERSITAT ROVIRA I VIRGILI
ROLE OF THE APOA5 GENE IN LIPID AND FAT-SOLUBLE VITAMIN METABOLISM
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ISBN:978-84-691-9478-2/DL:T-19-2009

Clinical Chemistry 52:10
1914–1919 (2006)

Lipids, Lipoproteins, and Cardiovascular Risk Factors

Protease Inhibitor-Associated Dyslipidemia in HIV-Infected Patients Is Strongly Influenced by the APOA5–1131T→C Gene Variation

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Background: **Hyperlipidemia associated with the protease inhibitor (PI) component of highly active antiretrovial treatment can lead to accelerated atherosclerosis. The apolipoprotein A-V (*APOA5*) gene, which affects VLDL production and lipolysis, may play a role in PI-induced hyperlipidemia, particularly in individuals with the APOA5–1131T→C genotype.**

Methods: **We measured lipoprotein changes in HIV-positive patients (n = 229) who had been followed for 5 years. For statistical analyses, we segregated the patients with respect to PI treatment and APOA5–1131T→C genotype.**

Results: **The frequency of the C allele was 0.08, similar to that in the general population. We found a strong effect of the APOA5–1131T→C genotype among patients receiving PIs. Carriers of the C allele had consistently increased mean (SD) triglyceride concentrations compared with noncarriers after 1 year [2.11 (1.62) vs 3.71 (4.27) mmol/L; *P* = 0.009], 2 years [2.48 (2.09) vs 4.02 (4.05) mmol/L, *P* = 0.050], 3 years [2.32 (1.71) vs 4.13 (4.26) mmol/L; *P* = 0.013], 4 years [2.90 (2.95) vs 5.35 (7.12) mmol/L; *P* was not significant], and 5 years [4.25 (5.58) vs 9.23 (9.63) mmol/L; *P* was not significant]. We observed the same effect on total cholesterol concentrations: after 1 year [4.93 (1.31) vs 5.87 (1.66) mmol/L; *P* = 0.006], 2 years [5.03 (1.12) vs 6.42 (2.48) mmol/L; *P* = 0.001], 3 years [5.11 (1.17) vs 6.38 (2.43) mmol/L; *P* = 0.009], 4 years [5.49 (1.71) vs 6.78 (3.03) mmol/L; *P* was not significant], and 5 years [5.56 (1.75) vs 7.90 (3.60) mmol/L; *P* was not significant]. HDL cholesterol showed a progressive reduction, leading to a considerably higher cholesterol/HDL cholesterol ratio after 3 years.**

Conclusion: **Variability in the *APOA5* gene predisposes patients with HIV, particularly those treated with PI, to severe hyperlipidemia.**

HIV-infected individuals have higher rates of subclinical atherosclerosis than the age-adjusted general population *(1)*, and the incidence of cardiovascular events is directly related to the years of exposure to antiretroviral therapy *(2, 3)*. The metabolic abnormalities associated with antiretroviral therapy *(4)*, chronic inflammatory status *(5)*, and the HIV infection itself *(6)* have been postulated as possible causes of this increased susceptibility to cardiovascular disease. Hypercholesterolemia and hypertriglyceridemia, which are well-established, independent, cardiovascular disease risk factors, are associated with the use of protease inhibitors, especially in patients undergoing ritonavir or ritonavir-boosted antiretroviral treatment. Despite similar antiretroviral treatment and demographic characteristics, however, not all HIV-infected patients develop these metabolic and cardiovascular complications. Hyperlipidemia, and more importantly, hypertriglyceridemia in HIV patients is highly influenced by genetic variability *(7)* and, among the candidate genes, the newly identified apolipoprotein A-V (*APOA5*)[1] has emerged as probably the most potent modulator of triglyceride (TG)[2] metabolism. The role of *APOA5* in regulating TG metabolism has been convincingly demonstrated in animal models *(8)* and in a large number of

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Received March 1, 2006; accepted July 18, 2006.

Previously published online at DOI: 10.1373/clinchem.2006.069583

[1] Human gene: *APOA5*, apolipoprotein A-V.

[2] Nonstandard abbreviations: TG, triglyceride; PI, protease inhibitor; HsCRP, high-sensitive C-reactive protein; SREBP, sterol regulatory element-binding proteins; BMI, body mass index.

association studies *(9)*, in which the –1131T→C is the commonly used variant. *APOA5* is expressed mainly in the liver and distributed predominantly on TG-rich lipoproteins such as chylomicrons and VLDL, and also on HDL. In human plasma, apo A-V is found at lower concentrations than other apolipoproteins. Its exact function has not been completely elucidated, but the available data indicate that it modulates TG metabolism by controlling production of VLDL and catabolism of the lipolysis of TG-rich lipoproteins *(10)*. The latter role has been confirmed not only in vitro but also in patients who develop severe hypertriglyceridemia attributable to apo A-V deficiency *(11)*.

Because protease inhibitor (PI)-associated dyslipidemia is caused by increases in VLDL production *(12)* and by impaired lipolysis *(13)*, we hypothesized that, in HIV-infected individuals, the *APOA5* gene could be an important indicator of predisposition to PI-related deterioration of the lipid profile. Hence, we analyzed lipid changes in HIV patients segregated with respect to treatment strategy and *APOA5* genotype.

Materials and Methods

PATIENTS

A total of 229 HIV-infected patients attending our outpatient clinic gave written informed consent to participate in the study. The present study is part of a longitudinal project in which atherosclerosis in HIV-infected patients is being assessed in a cohort of patients who are followed regularly in our outpatient clinic. The overall characteristics of these patients have been described previously *(14, 15)*. Time 0 of the study corresponds to commencement of therapy with PIs (n = 148) or without PIs (n = 81). Subsequent time-points are at 1, 2, 3, 4, and 5 years of treatment follow-up. Clinical and biochemical data and the APOA5–1131T→C genotype have been measured for all participants. Exclusion criteria were age <18 years and AIDS-related opportunistic disease when the study began. The Ethics and Investigation Committee of our Hospital approved the study.

LIPID PROFILE ANALYSES

We used standard laboratory methods to measure total cholesterol, HDL cholesterol, and TGs.

HIGH-SENSITIVE C-REACTIVE PROTEIN ANALYSES

We measured serum high-sensitive C-reactive protein (hsCRP) concentrations with a turbidimetric immunoassay (Biokit), according to the manufacturer's instructions.

APOA5 –1131T→C GENOTYPE ANALYSES

Genomic DNA was obtained from leukocytes and extracted with calibrated methods. The –1131T→C variation in the *APOA5* gene was detected with the oligonucleotide primers AV-1–5′-GAT TGA TTC AAG ATG CAT TTA GGA C-3′ and AV-2–5′CCC CAG GAA CTG GAG CGA AAT T-3′ to amplify a 187bp segment, and AV-2 primer forced a site for Mse I (New England Biolabs) enzymatic restriction. PCR was performed as described previously *(8)*.

STATISTICAL ANALYSES

We used a multivariate analysis on patients with complete data available [age, body mass index (BMI), sex, and lipid data at all time points] being entered into the analysis. To improve the statistical power of our dataset, we performed ANOVA analyses of data from every single time-point, used lipid values as a dependent variable, and normalized for confounding factors such as sex, age, BMI, and lipodystrophy. We also performed multivariate analyses of repeated measures, which confirmed the trends but did not reach statistical significance. For TG and hsCRP, calculations were performed on log-transformed values, although nontransformed concentrations are shown in the Tables and Figs.

We analyzed deviation from Hardy Weinberg equilibrium with the χ^2 goodness-of-fit test. Results are conveyed as mean (SD). Statistical significance was accepted at a value of $P < 0.05$.

Results

The data from a total of 229 HIV-infected patients were segregated according to APOA5 –1131T→C genotype and treatment scheme. For statistical purposes, the single patient homozygous for the C allele was pooled with those patients who were T/C heterozygote. For lipid analyses, patients undergoing the PI regimen (n = 148) and those not receiving PIs (n = 81) were studied separately.

The frequency of the C allele was 0.08, which is similar to that found in the Spanish general population (0.07) *(16)*. Allelic distribution was in Hardy-Weinberg equilibrium.

APOA5-ASSOCIATED CHANGES IN LIPIDS AND LIPOPROTEINS

The group of patients with the wild-type genotype (TT) and carriers of the rare variant (TC and CC) were comparable at baseline with respect to age, sex, immunologic status, hsCRP, total cholesterol, HDL cholesterol, and TGs (Table 1). Only BMI was considerably higher in the carriers of the wild-type allele.

PATIENTS RECEIVING PI THERAPY

Because hyperlipidemia is strongly associated with the PI regimen, we focused on the subgroup of 148 patients receiving PI as a component of their antiretroviral therapy, and we analyzed their lipid profile changes over the 5-year follow-up period. The 2 genotype groups were also comparable at baseline (pretreatment), including the percentage of patients receiving ritonavir (Table 1).

Carriers of the C allele had consistently higher TG concentrations than noncarriers at 1 year [2.11 (1.62) vs 3.71 (4.27) mmol/L; $P = 0.009$], 2 years [2.48 (2.09) vs 4.02

Table 1. Baseline selected epidemiological, immunological, and inflammatory characteristics of study participants segregated with respect to APOA5 genotype.

	All participants n = 229	APOA5 (TT) n = 191	APOA5 (TC+CC) n = 38	P	PI APOA5 (TT) n = 128	PI APOA5 (TC+CC) n = 20	P	No PI APOA5 (TT) n = 63	No PI APOA5 (TC+CC) n = 18	P
Age, years	38.6 (6.8)	38.5 (6.7)	40.1 (9.2)	NS[a]	39.3 (7.4)	39.7 (6)	NS	37.8 (5.4)	35 (8.7)	NS
Gender, % male	158 (62.9)	135 (69.9)	25 (65.7)	NS	92 (71.8)	16 (80)	NS	32 (51)	6 (33)	NS
BMI, kg/m^2	23 (3.1)	23.2 (3)	21.6 (2.9)	0.03	23 (2.8)	22.1 (3.4)	NS	23.6 (3.5)	21.5 (2.8)	0.05
CRP, mg/L	4 (5.6)	4 (5.7)	4.42 (5)	NS	4.2 (6.4)	5.9 (5.5)	NS	3.3 (3.4)	2.6 (3.8)	NS
Total cholesterol, mmol/L	4.60 (1.20)	4.60 (1.21)	4.58 (1.22)	NS	4.63 (1.31)	5.06 (1.02)	NS	4.53 (0.98)	3.98 (1.23)	0.04
Triglyceride, mmol/L	2.00 (2.05)	1.99 (2.15)	2.05 (1.47)	NS	2.14 (2.41)	2.17 (1.84)	NS	1.67 (1.41)	1.87 (0.75)	NS
HDL cholesterol, mmol/L	1.1 (0.4)	1.1 (0.4)	1.2 (0.2)	NS	1.1 (0.5)	1.3 (0.2)	NS	1.1 (0.3)	1.1 (0.2)	NS
Nadir CD4, cell/mm^3	344 (286)	343 (302)	449 (281)	NS	327 (321)	300 (309)	NS	340 (190)	453 (285)	NS
CD4 $<$200 mm^3, %	65 (28.1)	56 (29)	9 (23.6)	NS	44 (34.3)	6 (30)	NS	11 (16.9)	2 (11.1)	NS
HIV viral load, copies/mL	254 377 (793 524)	302 331 (938 393)	294 004 (508 390)	NS	351 107	NA	NS	144 467 (206 574)	159 354 (248 845)	NS
Lipodystrophy, %	20.9 (24.1)	37 (22.7)	10 (30.3)	NS	28 (25.2)	9 (47.4)	0.04	8 (12.3)	1 (5.5)	NS
Ritonavir boosted, %					33 (25.7)	7 (35)	NS			

Values are expressed as mean (SD). NS, not significant; NA, not applicable.

(4.05) mmol/L; $P = 0.050$], 3 years [2.32 (1.71) vs 4.13 (4.26) mmol/L, $P = 0.013$], 4 years [2.90 (2.95) vs 5.35 (7.12) mmol/L; P not significant], and 5 years [4.25 (5.58) vs 9.23 (9.63) mmol/L; P not significant], after adjustment of the data for age, sex, BMI, and the presence of lipodystrophy (Fig. 1).

Results were similar for total cholesterol. Carriers of the C allele had higher plasma cholesterol concentrations at 1 year [4.93 (1.31) vs 5.87 (1.66) mmol/L; $P = 0.006$], 2 years [5.03 (1.12) vs 6.42 (2.48) mmol/L; $P = 0.001$], 3 years [5.11 (1.17) vs 6.38 (2.43) mmol/L; $P = 0.009$], 4 years [5.49 (1.71) vs 6.78 (3.03) mmol/L; P = not significant], and 5 years [5.56 (1.75) vs 7.90 (3.60) mmol/L; P = not significant] (Fig. 2). HDL cholesterol concentrations showed a tendency toward decrease in carriers of the C allele and increase in patients with the wild-type alleles, but these differences did not reach statistical significance (data not shown).

The total cholesterol/HDL cholesterol ratio, which was 78% higher in carriers of the C allele than in carriers of the wild-type allele (Fig. 3), indicated that these lipid changes increased the risk of atherogenesis.

PATIENTS NOT RECEIVING TREATMENT WITH PIS

To investigate whether the effect of *APOA5* on the lipid profile in HIV patients was influenced by treatment with PIs or whether the effect was more generalized, we separately evaluated the 81 patients who were not receiving treatment with PIs. At baseline, the 2 genotype groups were comparable (Table 1). There were no differences between genotypes with respect to total cholesterol, TGs, HDL cholesterol, or the total cholesterol/HDL cholesterol ratio over the 5-year follow-up period (Figs. 1–3).

Discussion

Our results show that HIV-infected patients with the APOA5–1131C allele are predisposed to severe hyperlipidemia related to treatment with PIs, i.e., the adverse effects of PI appear to be exacerbated in patients with the C allele on the *APOA5* gene.

APOA5 ENHANCES PI-ASSOCIATED HYPERLIPIDEMIA

The PIs used in combined therapies can produce major fat redistribution, hyperlipidemia, and insulin resistance. These effects can be mitigated by replacing PIs with other antiretroviral drugs *(17)*. That these abnormalities do not develop in all patients on PI regimens suggests the involvement of genetic or environmental predisposing factors. We focused on the *APOA5* gene because it is probably the strongest genetic determinant of plasma TG *(9)* identified to date, and few data on the influence of *APOA5* on PI-induced hyperlipidemia are available.

Among the 229 HIV-patients we followed for a period of 5 years, those receiving the PI regimen tended, as expected, to have higher concentrations of total cholesterol and TGs during the treatment period. Despite similar baseline lipid values among all patients, however, the

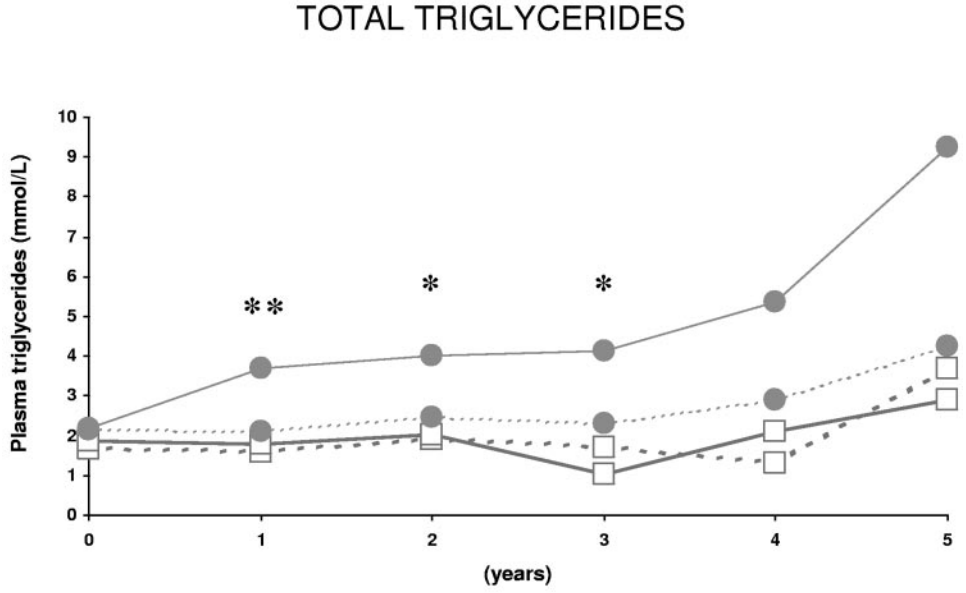

Fig. 1. Changes in plasma TG concentrations over the 5-year follow-up for carriers of the APOA5 –1131C allele (*solid line*) and carriers of the wild-type allele (*dotted line*) receiving either PI treatment (*closed circles*) or treatment not containing PIs (*open squares*).

Carriers of the C allele receiving PI treatment have considerably higher TG concentrations at 1 year [2.11 (1.62) vs 3.71 (4.27) mmol/L, $P = 0.009$], 2 years [2.48 (2.09) vs 4.02 (4.05) mmol/L, $P = 0.050$], and 3 years [2.32 (1.71) vs 4.13 (4.26) mmol/L, $P = 0.013$] of follow-up, representing 43%, 38%, and 44% of higher amounts in C allele carriers, respectively. Data were available for 15 carriers of the C allele and 96 wt treated with PI, and for 15 carriers of the C allele and 41 wt on alternative therapy.

individuals carrying the –1131C variant of the *APOA5* gene and undergoing treatment with PIs had the highest values at all of the follow-up time-points. Conversely, carriers of the –1131C variant not receiving PI treatment did not have such strongly increased concentrations of these lipids. This observation is in accordance with our previous studies demonstrating that the magnitude of the effect of the *APOA5* gene is more pronounced when conditions are more metabolically challenging *(16)*.

Increased cholesterol concentrations in carriers of the –1131C allele could also be the result of increased cholesterol delivery by the TG-rich lipoproteins, a well-described secondary feature of VLDL overproduction. Although detailed lipoprotein subfractionation was not available in the present group of patients, it is clear that increased VLDL synthesis, decreased catabolism, or a combination of these processes can lead to PI-induced hyperlipidemia. It is also clear that these metabolic perturbations are characterized essentially by an increasing TG component with a concomitant increase in the cholesterol concentration in these lipoproteins.

In our study sample, carriers of the APOA5 variant allele had higher rates of lipodystrophy. The *APOA5* gene may somehow predispose individuals to this lipid and fatty-tissue redistribution, but this hypothesis is not supported by our finding that the frequency of lipodystrophy among *APOA5* genotypes in the non-PI group did not differ from the frequency in the overall patient group.

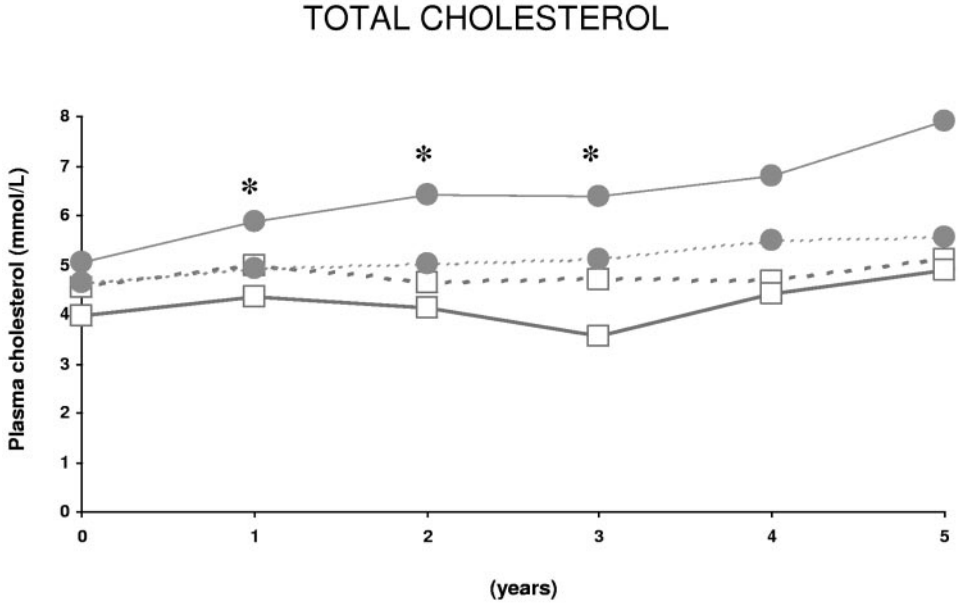

Fig. 2. Changes in plasma cholesterol over the 5-year follow-up are presented for carriers of the APOA5 –1131C allele (*solid line*) and carriers of the wild-type allele (*dotted-line*) receiving either PI treatment (*closed circles*) or treatment not containing PIs (*open squares*).

Carriers of the C allele receiving PI treatment have considerably higher total cholesterol concentrations at 1 year [4.93 (1.31) vs 5.87 (1.66) mmol/L, $P = 0.006$], 2 years [5.03 (1.12) vs 6.42 (2.48) mmol/L, $P = 0.001$], and 3 years [5.11 (1.17) vs 6.38 (2.43) mmol/L, $P = 0.009$] of follow-up, representing 16%, 21%, and 20% higher amounts in C allele carriers, respectively. Data were available for 15 carriers of the C allele and 96 wt treated with PI and for 15 carriers of the C allele and 41 wt on alternative therapy.

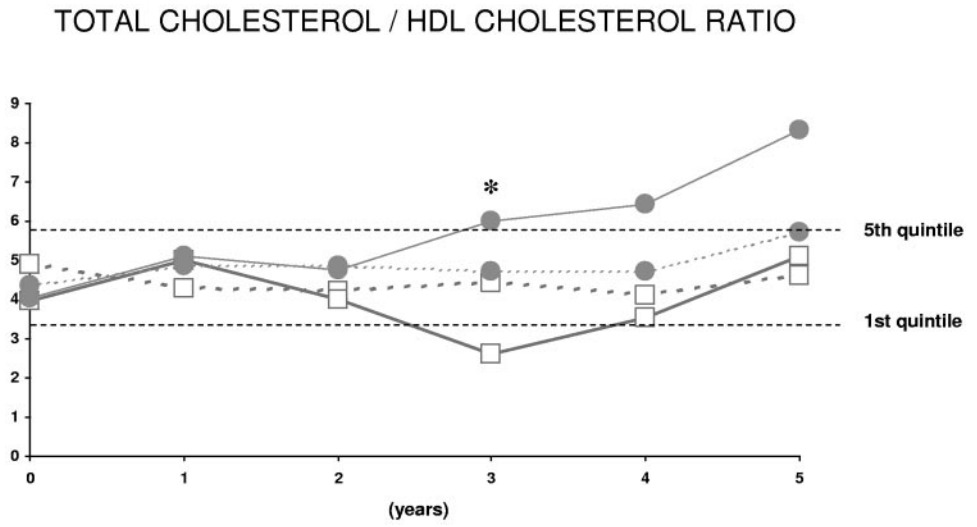

Fig. 3. Changes in plasma cholesterol/HDL cholesterol ratio over the 5-year follow-up are presented for carriers of the APOA5 –1131C allele (*solid line*) and carriers of the wild-type allele (*dotted-line*) receiving either PI treatment (*closed circles*) or treatment not containing PIs (*open squares*).

Although the 4 categories have comparable values at baseline, carriers of the –1131C allele on PI therapy have a considerably higher ratio (above the 5th quintile) after 3 years of follow-up.

Conversely, after adjustment for the presence of lipodystrophy, all of the observed differences with respect to lipid concentrations remained considerable, indicating that the hyperlipemic effect was truly associated with the *APOA5* gene and was not influenced by the presence of lipodystrophy.

A mechanistic approach to this observation could be that, although the exact function in vivo of apo A-V is not known, in vitro studies suggest that it acts by decreasing the assembly of the VLDL particle *(18)* and its secretion and by stimulating lipolysis in the circulation *(10)*. Conversely, PI-induced hyperlipidemia has been shown to be caused by an increase in VLDL production *(12)* as well as by impaired lipolysis *(13)* as a consequence, in part, of a direct interaction between the PI and the sterol regulatory element-binding proteins (SREBP)1 and 2, leading to an accumulation of SREBPs in the nucleus, which stimulates lipid synthesis *(19)*. Because APOA5 expression is downregulated by SREBP1c *(20)*, we assume that apo A-V plays a determining role in PI-induced hyperlipidemia. Whether the –1131C allele is a functional variant or is acting as a marker of a functional variant elsewhere in the gene is not clear *(21)*.

APOA5 and atherogenic lipid profile

Patients who are receiving PI-treatment and who carry the –1131C allele present with a lipoprotein profile that deteriorates rapidly because of increasing of total cholesterol and TGs and a decrease in the HDL fraction, with the atherogenic total/HDL cholesterol ratio reaching the highest quintile after 3 years in carriers of the mutant C allele *(22)*. This scenario does not occur in patients who are receiving the same treatment but who carry the wild-type gene or who are not receiving PI therapy.

One of the limitations of our study is that there were fewer patients available to follow up at years 4 and 5 than for the first 3 years because 4 to 5 years ago there was less concern regarding lipid alterations in individuals with HIV-AIDS, so the percentage of individuals with HIV-AIDS being treated for hyperlipidemia was considerably lower in the first 2 years of recruitment into the present study. We suspect that the patients who were recruited were those with more evident dyslipidemia, which might explain the greater increase in plasma TG concentrations in year 5. It is important to note, however, that all the main conclusions of the study were drawn from the data obtained during 1–3 years of follow-up. Data from 4–5 years of follow-up were included for completeness, and to indicate that the trends toward increased lipids observed in the first 3 years continued in the same direction over the subsequent years of follow-up in those patients, for whom detailed lipid datasets were available.

In conclusion, our results indicate that variability at the *APOA5* gene variation predisposes HIV patients, particularly those treated with PIs, to severe hyperlipidemia. Although these results must be confirmed in future studies, they suggest the possibility of using the *APOA5* gene as a marker of predisposition to severe hyperlipidemia as a consequence of treatment with PIs. In HIV-positive individuals found to carry this variation, treatment alternatives that exclude PI may need to be considered.

This study was supported by grants from the Instituto de Salud Carlos III, Red de Centros de Metabolismo y Nutrición (C03/08), genetic hyperlipidemias (G03/181), Mediterranean diet (G03/140), and SAF-2002-02781 (Madrid, Spain). Dr. Blai Coll is in receipt of a career development award from the Instituto de Salud Carlos III (Madrid, Spain). Montse Guardiola is a recipient of a predoctoral fellowship at the Spanish Ministry of Science and Technology (BES-2003-1090).

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ISBN:978-84-691-9478-2/DL:T-19-2009

La grasa saturada en la dieta disminuye la expresión hepática de *APOA5* en ratones deficientes en apo E

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Introducción. En el desarrollo de la arteriosclerosis intervienen numerosos factores; en especial la edad, la dieta y la hiperlipidemia. La apolipoproteína (apo) A-V desempeña un papel destacado en el control del metabolismo lipídico. Nuestro objetivo es estudiar en ratones hiperlipémicos el efecto que la grasa de la dieta tiene en la expresión hepática del gen de la apo A-V *(APOA5)* y su relación con el desarrollo de la arteriosclerosis y sus factores de riesgo.

Material y métodos. Utilizamos 72 ratones *knock-out* para el gen de la apo E *(KO-APOE)* separados en 3 grupos (n = 24): los que recibían dieta convencional de ratón o dieta rica en grasa saturada (20% aceite de palma) sola o suplementada con 0,25% de colesterol. Las muestras se tomaron a las 16, 24 y 32 semanas de edad. Las determinaciones analíticas incluyeron parámetros lipídicos e inflamatorios, la superficie de lesión arteriosclerótica en la aorta y la expresión de *APOA5* en hígado.

Resultados. La ingesta de dieta rica en grasa saturada disminuye un 48% (p = 0,001) de media la expresión hepática de *APOA5* y la suplementación con colesterol revierte este efecto. Estos efectos se observaron a las diferentes edades de los ratones. La expresión hepática de *APOA5* aumenta significativamente (p < 0,0001) en función de la edad, el número de lesiones arterioscleróticas en la aorta y el grado de inflamación en los ratones.

Conclusiones. La grasa saturada de la dieta disminuye significativamente la expresión hepática de *APOA5*, que a su vez aumenta con la edad a todas las dietas suministradas y se correlaciona con el área ateromatosa y el estado inflamatorio.

Palabras clave:
APOA5. KO-APOE. Triglicéridos. Dieta. Arteriosclerosis. MCP-1.

SATURATED FAT FROM DIET DOWNREGULATES *APOA5* HEPATIC EXPRESSION IN APO E DEFICIENT MICE

Introduction. Many factors are involved in atherosclerosis development, especially age, diet and hyperlipidemia. Apolipoprotein (apo) A-V plays a key role in the control of lipid metabolism. The aim of this study was to determine the effect of dietary fat intake on hepatic expression of the apo A-V gene *(APOA5)* in hyperlipidemic mice and its association with risk factors for atherosclerosis and atherosclerosis development.

Material and methods. We used 72 knock-out mice for the apo E gene (KO-APOE) divided in three groups (n=24) that received a chow diet, a diet rich in saturated fat (20% palm oil) alone, or a diet supplemented with 0.25% of cholesterol. Samples were obtained at 16, 24, and 32 weeks. Laboratory determinations included lipid and inflammatory parameters, area of atherosclerotic

Este estudio se ha llevado a cabo gracias a un proyecto financiado por las Becas FEA/SEA 2004 para Investigación básica en el tema "Aspectos básicos de la arteriosclerosis y sus factores de riesgo"; un proyecto subvencionado por el Fondo de Investigación Sanitaria FIS (PI020032) y una beca predoctoral del Ministerio de Ciencia y Tecnología (BES-2003-1090).

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Recibido el 9-11-2006 y aceptado el 28-11-2006.

lesions in the aorta, and *APOA5* expression in the liver.

Results. Intake of a saturated fat-rich diet reduced mean hepatic expression of *APOA5* by 48% (*P*=0.001), while cholesterol supplementation reversed this effect. These effects were found at the different ages of mice. Hepatic *APOA5* expression significantly increased (*P*=0.001), depending on age, the number of atherosclerotic lesions in the aorta, and the degree of inflammation in these mice.

Conclusions. Saturated dietary fat significantly downregulates hepatic *APOA5* expression, which also increases with age, in all the diets administered and correlates with atheromatous area and inflammatory status.

Key words:
APOA5. *APOE*-knock-out. TC. Diet. Arteriosclerosis. MCP-1

Introducción

La arteriosclerosis en humanos es un proceso de desarrollo lento y es la causa principal de las enfermedades cardiovasculares y cerebrovasculares, que causan una elevada mortalidad y morbilidad en los países desarrollados. La hiperlipidemia, la inflamación, la oxidación y la nutrición son factores clave en la aparición y progresión de la arteriosclerosis.

Debido a la dificultad de estudiar la arteriosclerosis en humanos, el ratón deficiente para la apolipoproteína E (apo E) ha sido muy utilizado como modelo gracias a que su metabolismo lipídico se parece mucho al de los humanos y a que el desarrollo de su proceso arteriosclerótico es rápido y reproducible. Además, si estos ratones se alimentan con una dieta enriquecida en colesterol y grasas, los valores de colesterol plasmático y la aterogénesis aumentan de manera proporcional[1].

Las alteraciones del metabolismo de las lipoproteínas son un factor de riesgo para la arteriosclerosis y pueden producirse como resultado de errores en la regulación de las apolipoproteínas. En el cromosoma 11 humano existe una región que agrupa los genes de las apolipoproteínas A-I/C-III/A-IV/A-V, que ha sido muy estudiada por su implicación en el metabolismo lipídico. El último de ellos *(APOA5)* se ha descubierto recientemente y ha demostrado tener una influencia especialmente relevante en el control de la trigliceridemia. Los animales que sobreexpresan el gen tienen un 60% menos triglicéridos (TG) circulantes, y en los animales que carecen del gen la trigliceridemia aumenta hasta 4 veces[2]. El gen *APOA5* se expresa únicamente en el hígado, donde se postula que reprime la síntesis y/o secreción de las lipoproteínas de muy baja densidad (VLDL)[3]. Estudios in vitro demuestran que la apo A-V estimula la actividad hidrolítica de la lipoproteína lipasa unida al endotelio[4]. En los últimos años, varios grupos han descrito (mediante estudios de asociación) que los polimorfismos en el gen *APOA5* se asocian a mayores concentraciones de TG en plasma[5], aunque su relación con el riesgo cardiovascular es todavía controvertida[6-8]. Otros estudios describen una asociación entre polimorfismos del gen *APOA5* y la dieta y sugieren que su expresión está regulada por la alimentación[9,10], aunque este punto no ha sido demostrado.

Nuestro objetivo ha consistido en estudiar el efecto que produce la dieta en la expresión de *APOA5* en un modelo experimental de arteriosclerosis y la influencia de los cambios inducidos en *APOA5* sobre parámetros lipídicos, la propia lesión arteriosclerótica y las concentraciones circulantes del marcador inflamatorio clave en ratones, el *monocyte chemotactic protein -1* (MCP-1).

Material y métodos

Ratones KO-apoE y diseño del estudio

La experimentación con los animales se llevó a cabo en las condiciones que establece la normativa legal vigente (Real Decreto 223/1998) y la tutela de la Comisión de Experimentación científica en animales de nuestra universidad.

Se utilizó 72 ratones macho KO-apoE agrupados en jaulas a 21-23 °C, con un grado de humedad del 50-60%, sometidos a un ciclo de 12 h de luz/oscuridad y con libre acceso tanto al agua como a la comida.

Los ratones se dividieron en 3 grupos de estudio que recibieron dieta convencional de ratón (pobre en grasa y sin colesterol. BK Universal, Barcelona) o dieta rica en grasas saturadas (20% aceite de palma. Unilever, Bilbao) con diferente contenido en colesterol (0 y 0,25% p/p. Sigma Chemical Co., St. Louis, MO, Estados Unidos) y se han sacrificado a las 16, 24 y 32 semanas de edad. Este diseño nos permite estudiar la expresión del gen *APOA5* en relación con la dieta y con la edad y, por tanto, observar patrones temporales.

Obtención de muestras

Durante el procedimiento experimental, se obtuvo muestras de sangre mediante punción en la cola y semanalmente se realizó el control del peso de todos los animales. Inmediatamente antes del sacrificio, se administró a los animales 0,1 ml de una mezcla de ketamina/xilacina para anestesiarlos por vía intraperitoneal. Una vez sacrificados, se obtuvo sangre mediante punción ventricular y también se extrajo el hígado y el corazón, que se congelaron inmediatamente a –80 ºC, hasta su posterior utilización.

Determinaciones lipídicas

Se determinaron los valores de colesterol y TG en plasma mediante técnicas estándar de laboratorio. Para caracterizar la composición lipídica de la esteatosis presente en estos animales, se determinó los contenidos de colesterol y TG en homoge-

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nizados de tejido hepático después de la extracción de lípidos con isopropilo alcohol-hexano[11].

Cuantificación de la lesión ateromatosa

La cuantificación de la lesión ateromatosa se determinó según el método de Tangirala et al[12]. Después del sacrificio, se congeló inmediatamente el corazón junto con el origen de la aorta; los cortes seriados obtenidos mediante un criostato se han teñido con un colorante con afinidad por los lípidos, el Sudan IV. La imagen microscópica para medir las áreas teñidas con este colorante, correspondientes a las áreas de lesión ateromatosa, se capturó mediante una cámara de vídeo y la extensión de la arteriosclerosis se ha cuantificado con un sistema de análisis de imagen automatizado (AnalySIS Soft Imaging System, Münster, Germany).

Concentración plasmática de MCP-1

La concentración plasmática del marcador inflamatorio MCP-1 se ha determinado utilizando un *kit* de ELISA (Mouse MCP-1 ELISA kit, Pierce, Rockford, Estados Unidos), siguiendo las instrucciones del fabricante.

Extracción de ARN y PCR a tiempo real

La extracción del ARN total de tejido hepático se realizó mediante el equipo de extracción ABI PRISM 6100 Nucleic Acid PrepStation de Applied Biosystems siguiendo las instrucciones del fabricante. La pureza y la concentración del ARN se han estimado mediante la relación de absorbancia 260 nm/280 nm. Se ha retrotranscrito 1 μg de ARN a ADNc siguiendo las instrucciones del fabricante y utilizando el termociclador PE Biosystems 2400.

La expresión del ARN mensajero (ARNm) de *APOA5* se ha cuantificado mediante reacción en cadena de la polimerasa (PCR) a tiempo real utilizando el equipo ABI Prism 5700 Sequence Detector System (Applied Biosystems, Foster City, Estados Unidos), combinado con la utilización de *primers* y sondas Taqman®. Éstos se obtuvieron de Applied Biosystems como productos Assays-on-Demand validados y prediseñados. El ARN ribosomal (ARNr) A 18S se utilizó como control endógeno y los valores de expresión se calcularon de acuerdo con el método de ΔΔct (ABI Prism 7700 Sequence Detection System. User bulletin n.º 2. Revisión A. Foster city [CA]: Applied Biosystems, 1997).

Análisis estadístico

Los datos se expresan como media ± desviación estándar. La comparación de medias se ha determinado mediante la prueba de ANOVA univariante y para las variables categóricas se utilizó la prueba de la χ^2.

Las correlaciones entre la expresión de *APOA5*, la extensión de la lesión en la aorta y la concentración plasmática del parámetro inflamatorio MCP-1 se han determinado mediante regresión lineal.

Resultados

Efecto de la dieta sobre los lípidos y la arteriosclerosis

En la tabla 1 se muestra cómo la administración de dieta rica en grasa saturada (20% aceite de palma), al suplementarla con colesterol, hace aumentar significativamente los valores circulantes de colesterol respecto a la dieta convencional en los ratones KO-apoE ($p < 0,0001$) y no modifica las concentraciones circulantes de TG. La suplementación con colesterol también produce un aumento significativo de los valores de colesterol y TG en el hígado ($p < 0,0001$ y $p = 0,013$, respectivamente).

La superficie de lesión arteriosclerótica medida en la aorta y la presencia de esteatosis en el hígado aumentan significativamente con el contenido en grasa de la dieta ($p < 0,0001$) (fig. 1). La concentración plasmática de MCP-1 también tiende a aumentar, pero este incremento no es estadísticamente significativo.

La ingesta de grasa saturada disminuye la expresión hepática del gen APOA5

Concretamente, la adición de un 20% de aceite de palma disminuye los valores de ARNm de *APOA5* en el hígado en un 75% ($p = 0,020$) en los ratones de 16 semanas, un 35% (p = NS) en los de 24 semanas y un 45% ($p = 0,023$) en los de 32 semanas, respecto a la dieta convencional. Sin embargo, la adición de 0,25% de colesterol a la dieta rica en grasa saturada evita esta disminución en los valores de ARNm de *APOA5* que no difieren de los de la dieta convencional tanto en los ratones de

Tabla 1. Valores basales de parámetros lipídicos y características de todos los ratones según el tipo de dieta

	DN	DRG	DRG + 0,25% COL	p
COL (mmol/l)	7,9 ± 2,4	12,4 ± 2,1	20,4 ± 4,4	< 0,0001
TG (mmol/l)	0,8 ± 0,3	0,9 ± 0,2	0,8 ± 0,4	NS
COL en hígado (nmol/l)	4,8 ± 1,0	4,8 ± 0,9	7,6 ± 2,5	< 0,0001
TG en hígado (nmol/l)	5,6 ± 1,4	6,2 ± 2,9	9,4 ± 5,6	0,013
Cuantificación de la lesión arteriosclerótica (Um^2)	149.691,0 ± 131.747,4	365.996,3 ± 273.294,0	1.117.418,3 ± 734.723,5	< 0,0001
MCP-1 (pg/ml)	161,9 ± 63,4	200,1 ± 114,8	236,2 ± 118,4	NS
Presencia de esteatosis (% de ratones)	0	14,3	85,7	0,005

COL: colesterol; DN: dieta convencional; DRG: dieta rica en grasa saturada (20% aceite de palma); MCP 1: *monocyte chemotactic protein-1;* NS: no significativo; TG: triglicéridos.
Los resultados se presentan como media ± desviación estándar.

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ISBN:978-84-691-9478-2/DL:T-19-2009

16 semanas (p = 0,003) como en los de 32 (p = 0,039) (fig. 2).

Efecto de la edad sobre los lípidos y la arteriosclerosis

La tabla 2 muestra las diferencias entre los parámetros lipídicos determinados en los ratones alimentados con todas las dietas, según la edad a la que fueron sacrificados.

Vemos que con la edad los valores circulantes de colesterol no presentan ningún cambio y que los valores de TG circulantes disminuyen significativamente a las 32 semanas (p = 0,016). Los valores de colesterol en el hígado tienden a disminuir con la edad y en cuanto a los TG vemos que disminuyen significativamente (p = 0,003). El área de la lesión arteriosclerótica aumenta, tal y como esperábamos, así como el grado de inflamación determinado con la concentración de MCP-1 en plasma (p < 0,0001 y p < 0,0001, respectivamente). Con la edad también aumenta el número de ratones que presentan esteatosis (p = 0,037).

La expresión hepática del gen APOA5 *aumenta con la edad*

Los valores hepáticos de ARNm de *APOA5* se correlacionan positivamente con la edad (R^2 = 0,405; p < 0,0001) y negativamente con los valores de colesterol y TG en el hígado (R^2 = 0,082; p = 0,047; y R^2 = 0,087; p = 0,040, respectivamente). Los ratones

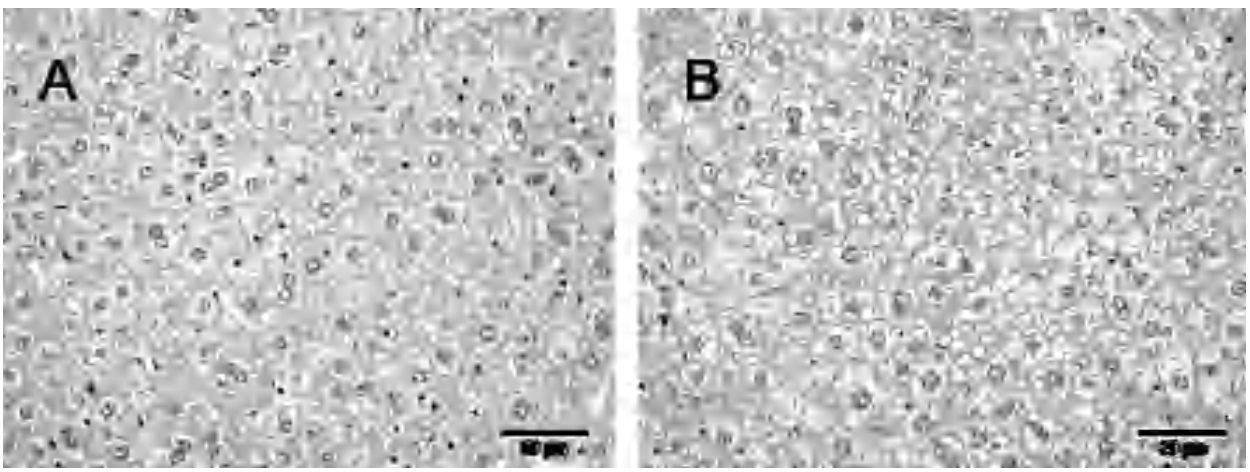

Figura 1. Esteatosis en hígado según el tipo de dieta.
Secciones de tejido hepático de ratón KO-apoE de 32 semanas de edad alimentado con dieta convencional (A) y de ratón alimentado con dieta rica en grasa saturada (B) donde se observa una mayor presencia de microvesículas lipídicas.

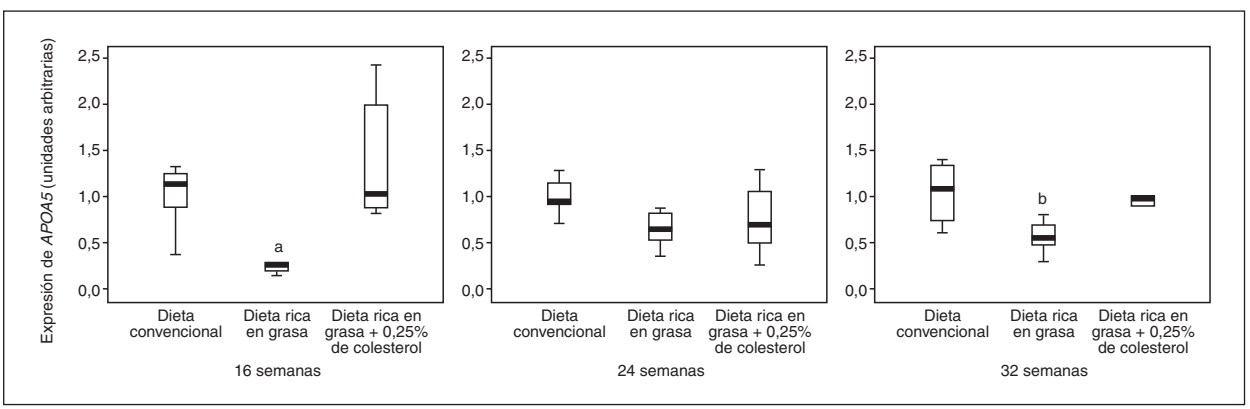

Figura 2. Distribución de los valores de expresión hepática de *APOA5* en relación con el tipo de dieta que recibían los ratones agrupados por edad. La expresión hepática de *APOA5* en los ratones alimentados con dieta rica en grasa saturada está significativamente disminuida respecto de la que presentan los ratones alimentados con dieta convencional o dieta rica en grasa saturada suplementada con colesterol en los ratones de 16 (p = 0,020)[a], 24 (p = NS) y 32 (p = 0 ,023)[b] semanas de edad, respectivamente.

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Tabla 2. Valores basales de parámetros lipídicos y características de todos los ratones a las diferentes edades de sacrificio

	16 semanas	24 semanas	32 semanas	p
COL (mmol/l)	13,1 ± 5,3	12,8 ± 5,1	14,2 ± 7,2	NS
TG (mmol/l)	0,9 ± 0,3	0,9 ± 0,4	0,6 ± 0,1	0,016
COL en hígado (nmol/ml)	5,8 ± 1,9	6,3 ± 2,8	5,1 ± 1,4	NS
TG en hígado (nmol/ml)	7,8 ± 3,4	8,6 ± 5,5	4,7 ± 1,8	0,03
Cuantificación de la lesión arteriosclerótica (Um^2)	137.372,3 ± 80.361,0	573.481,8 ± 507.980,2	895.630,4 ± 744.224,9	0,001
MCP-1 (pg/ml)	132,7 ± 46,2	293,4 ± 133,2	211,0 ± 87,5	0,001
Presencia de esteatosis (%)	0	71,4	28,6	0,037

COL: colesterol; MCP-1: *monocyte chemotactic protein 1;* NS: no significativo; TG: triglicéridos.
Los resultados se presentan como media ± desviación estándar.

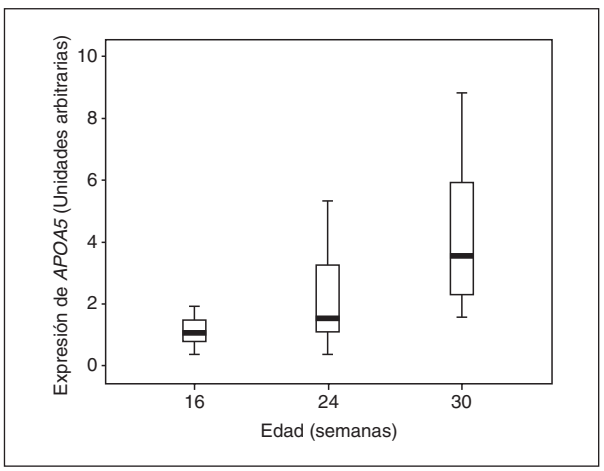

Figura 3. Distribución de los valores de expresión hepática de *APOA5* en relación con la edad a la que fueron sacrificados los ratones. Los ratones de 32 semanas de edad presentaban mayores valores de expresión de *APOA5* en hígado respecto de los ratones de 16 semanas ($p < 0,001$).

de 24 y 32 semanas de edad presentan unos valores de media del 89,1 y el 282,8% superiores respecto a la expresión que presentan los de 16 semanas (fig. 3). Este efecto es independiente de la dieta ya que también se observa si separamos los ratones en los diferentes grupos de dieta.

Arteriosclerosis

Para intentar comprender el alcance de los cambios en *APOA5* inducidos por las distintas dietas, analizamos las correlaciones entre los valores de ARNm de *APOA5* y de lípidos, la lesión ateromatosa y el grado de inflamación en estos ratones.

La expresión hepática de *APOA5* no se correlaciona significativamente con ningún parámetro lipídico determinado en los ratones.

Tal y como muestran las figuras 4 y 5, observamos que la expresión de *APOA5* tiende a correlacionarse positivamente con la superficie de lesión en la arteria aorta de los ratones alimentados con dieta rica en grasa saturada, y negativamente en los ratones alimentados con dieta convencional o dieta rica en grasa saturada suplementada con colesterol. En el caso de la concentración plasmática de MCP-1, también observamos el mismo comportamiento de forma positiva y significativa en los ratones alimentados con dieta rica en grasa saturada y correlacionando negativamente en los ratones alimentados con los otros 2 tipos de dietas.

Discusión

Nuestros resultados demuestran que la grasa saturada disminuye cerca de un 50% la expresión hepática de *APOA5* en el ratón KO-apoE. Además, aumenta con la edad en todas las dietas administradas.

Dieta

La expresión hepática del gen *APOA5* disminuye significativamente en todos los ratones de diferentes edades alimentados con dieta rica en ácidos grasos saturados; sin embargo, al suplementar esta dieta con 0,25% de colesterol tal efecto desaparece.

Los modelos animales indican que la sobreexpresión de *APOA5* ayuda a controlar los valores plasmáticos de TG[13]. Por lo tanto, la disminución de hasta un 75% en animales jóvenes de la expresión hepática de *APOA5* supondría un efecto adverso en los TG. Ello estaría de acuerdo con los estudios que muestran que una dieta rica en grasa saturada aumenta los valores de TG y que el déficit de apo A-V en humanos está asociado a hipertrigliceridemia grave[14,15]. Sorprendentemente, no hemos observado que la disminución de *APOA5* afecte a los valores de TG. Creemos que ello puede ser una característica específica de los ratones KO-apoE en los que sabemos que la vía secretora de TG en los hepatocitos está alterada[16], lo cual podría restar influencia a *APOA5* en la síntesis de VLDL.

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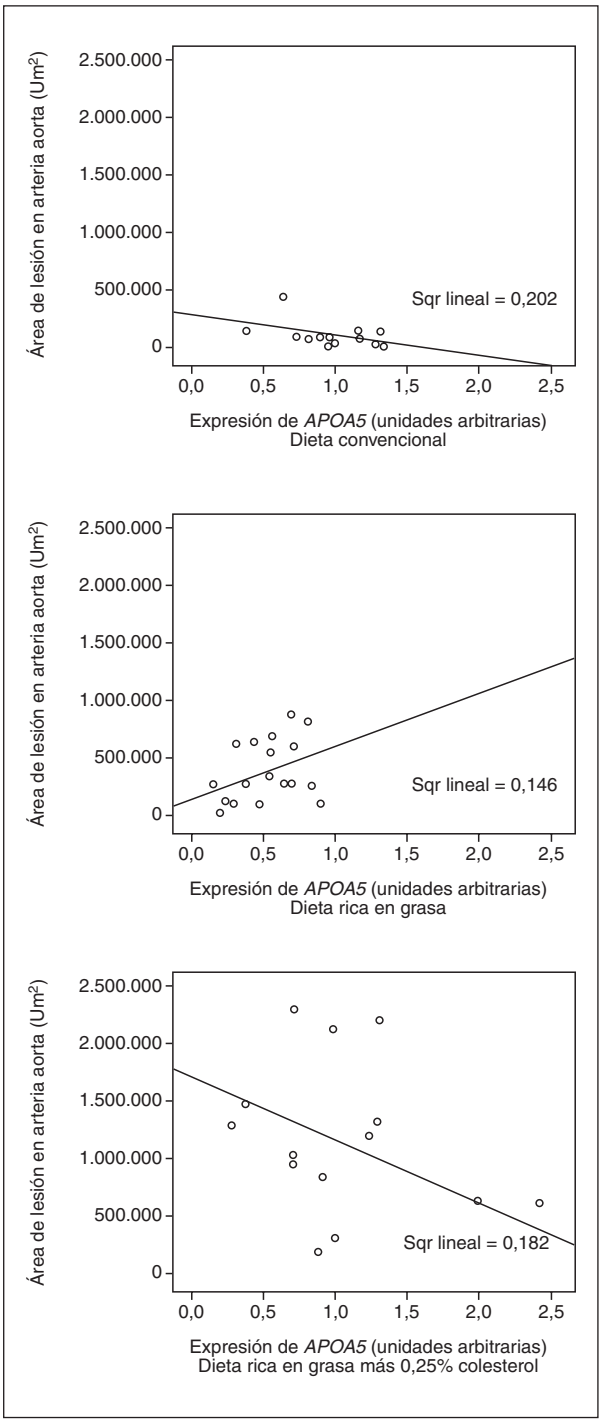

Figura 4. Correlación entre la expresión hepática de *APOA5* y la cantidad de lesión en la arteria aorta según el tipo de dieta con que estaban alimentados los ratones. Los ratones alimentados con dieta convencional presentan una correlación negativa con la superficie de lesión en la arteria aorta (R^2 = 0,202; p = NS), al igual que los alimentados con dieta rica en grasa más 0,25% colesterol (R^2 = 0,182; p = NS). En cambio, los ratones alimentados con dieta rica en grasa saturada tendían a presentar una correlación positiva con el área de la lesión (R^2 = 0,146; p = NS).

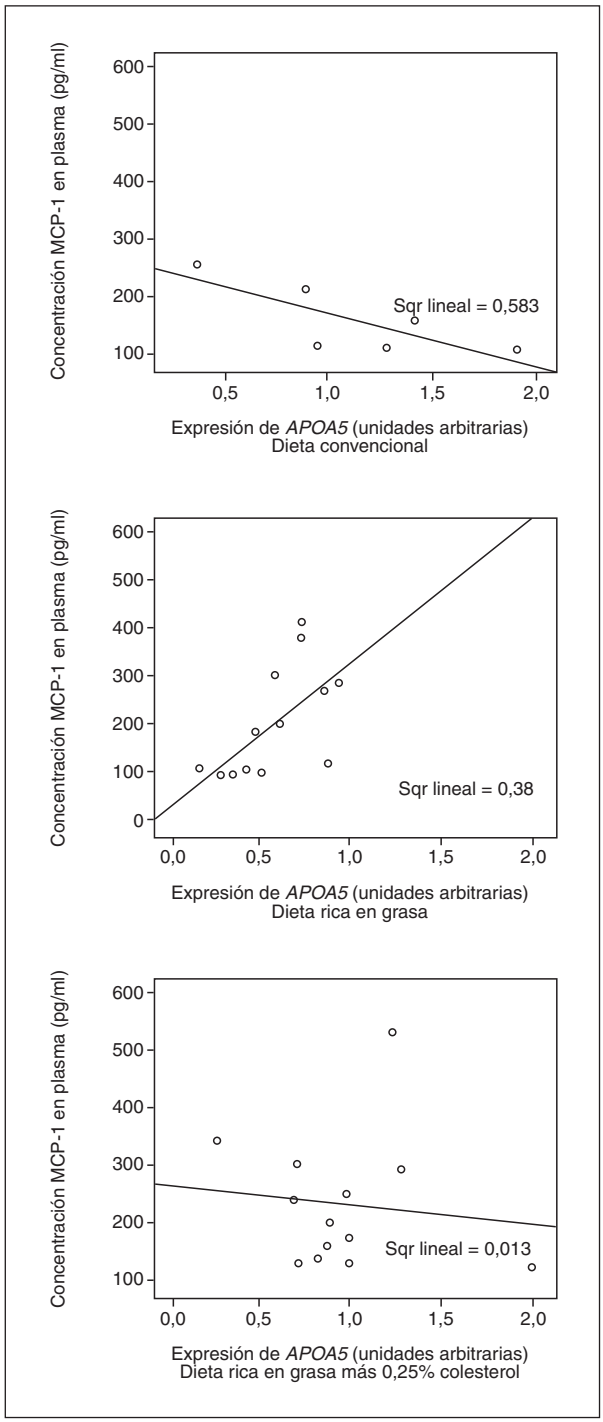

Figura 5. Correlación entre la expresión hepática de *APOA5* y la concentración plasmática de *monocyte chemotactic protein 1* (MCP-1) según el tipo de dieta con que estaban alimentados los ratones. Los ratones alimentados con dieta convencional tendían a presentar una correlación negativa con la concentración de MCP-1 en plasma (R^2 = 0,583; p = NS), al igual que los alimentados con dieta rica en grasa más 0,25% colesterol (R^2 = 0,013; p = NS). En cambio, los ratones alimentados con dieta rica en grasa saturada presentaban una correlación positiva con el área de la lesión (R^2 = 0,380; p = 0,025).

El mecanismo mediante el cual los ácidos grasos libres y el colesterol actuarían en *APOA5* no está claro; sin embargo, sabemos que los ácidos grasos regulan la expresión de genes implicados en el metabolismo energético y en este proceso de regulación también están implicados varios tipos de receptores nucleares encargados del reconocimiento de secuencias en la zona reguladora del gen. En el caso de *APOA5,* sabemos que está regulado, entre otros, por receptor de la activación de peroxisomas α (PPARα)[17], que tiene como ligandos naturales los ácidos grasos, y nuestros resultados describen por primera vez un papel regulador de los ácidos grasos saturados en la expresión de *APOA5*.

El efecto de la suplementación de colesterol en la expresión hepática de *APOA5* puede explicarse teniendo en cuenta que su expresión está estimulada por el receptor nuclear RORα[18,19], cuya actividad transcripcional está regulada por el colesterol. Está descrito que las condiciones que afectan al contenido celular de colesterol pueden modular la actividad de RORα in vitro[20], por tanto, es de esperar que el tipo de dieta que administramos en estos ratones, que aumenta los valores de colesterol, hace que RORα sea constitutivamente activo y, por tanto, estimule la expresión de *APOA5*, por lo que se pierde el efecto producido por la dieta rica en grasa saturada.

Edad

El ratón KO-apoE alimentado con dieta aterogénica experimentan un deterioro progresivo del hígado (esteatosis e inflamación). La expresión hepática del gen *APOA5* está significativamente aumentada con la edad y este efecto no depende del tipo de dieta recibida. Sin embargo, debido al deterioro funcional del hígado de estos animales sometidos a dietas aterogénicas, creemos que este aumento puede deberse a este hecho más que a la edad per se. Además, los cambios en la expresión de *APOA5* debidos a la edad se correlacionan de forma negativa y significativa con los valores de colesterol y TG en el hígado. Ello es congruente con el hecho de que *APOA5* es uno de los genes más sobreexpresados durante el proceso de regeneración hepática y que ya ha sido considerado como proteína de fase aguda[21,22].

Arteriosclerosis

Desde que se descubrió en 2001 el gen *APOA5* se ha asociado a mayores valores de TG circulantes en varios tipos de poblaciones como diabéticos e hiperlipémicos; nuestro grupo ha descrito asociación en pacientes con HLFC y en pacientes infectados por el virus de la inmunodeficiencia humana tratados con inhibidores de la proteasa[23-25]. También se ha asociado a mayor riesgo de enfermedad cardiovascular y se ha identificado como proteína de fase aguda, un efecto independiente de los valores plasmáticos de TG.

Como ya se ha comentado, el descenso en la expresión hepática de *APOA5* debido a la ingesta de dieta rica en grasa saturada no se correlacionaba con los valores de lípidos, posiblemente debido a la alteración en la vía secretora hepática de TG en los ratones KO-apoE[16]. Por ello nos interesó saber si este efecto se correlacionaba con otros parámetros implicados con el proceso arteriosclerótico determinados en estos ratones.

La expresión hepática de *APOA5* se correlaciona con parámetros inflamatorios como la concentración en plasma de MCP-1 y con la superficie de lesión arteriosclerótica. Estas correlaciones varían según el tipo de dieta con que alimentamos a los ratones, un hecho que refleja la importancia del efecto modulador de la dieta en la enfermedad cardiovascular y sus factores de riesgo. Según el tipo de dieta, observamos que los valores de expresión hepática de *APOA5* presentan un patrón de expresión inverso. Con la dieta convencional de ratón, los valores de expresión de *APOA5* se correlacionan negativamente con la cantidad de lesión arteriosclerótica y con el grado de inflamación presente en el ratón, lo que sugiere cierta asociación con el riesgo cardiovascular. Con la dieta rica en grasa saturada vemos que se correlaciona positivamente, tanto con el grado de arteriosclerosis como con el grado de inflamación de los ratones, y probablemente actúa como proteína de fase aguda en condiciones de estrés del organismo.

En conjunto, los datos son congruentes con una doble función del gen de *APOA5*. Como regulador del metabolismo de los lípidos, en relación con la síntesis y secreción de lipoproteínas y el control de las reservas hepáticas de grasa, y como proteína de fase aguda con unos valores más elevados de actividad transcripcional, asociados a parámetros inflamatorios.

En resumen, la grasa saturada de la dieta disminuye cerca de un 50% la expresión hepática de *APOA5* en el ratón KO-apoE, y por tanto, puede representar una forma de modificar la expresión de *APOA5* en el hígado.

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THE APOA5 GENE IS EXPRESSED IN HUMAN INTESTINAL TISSUE AND RESPONDS TO FATTY ACID AND FIBRATE EXPOSURE *IN VITRO*

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Key words: apolipoprotein, triglycerides, intestine, A1/C3/A4/A5

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1. Summary

APOA5 is a key gene regulating TG levels. The protein product apo A-V is reported to be secreted exclusively in the liver where it may regulate TG-rich particle synthesis and secretion. Since the same process occurs in the intestine, we postulated that it would also express *APOA5*. We report here the identification of mRNA expression of *APOA5* in the intestine, being the protein concentration approximately one third that in the liver. *APOA5* was expressed mainly in duodenum and colon, and was inversely correlated with *APOC3* expression. In the human intestinal TC-7 cell line, *APOA5* expression was downregulated by mono- and poly-unsaturated fatty acids and upregulated by saturated fatty acids. Short-chain fatty acid butyrate increased *APOA5* expression almost 4-fold while *APOB* was downregulated by increasing butyrate concentrations. When TC-7 cells were incubated with PPARα agonist, *APOA5* expression was increased by 60%, while the expression of *APOB*, *MTP* and *APOC3* were decreased by 50, 30 and 45%, respectively. Our results demonstrate that *APOA5* is expressed in the intestine and not, as previously thought, only in the liver. Further, its expression is modifiable by dietary and pharmacological stimuli.

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2. Introduction

Alterations in the apolipoprotein (apo) A1/C3/A4 gene cluster on human chromosome 11 have been tightly linked to the control of TG-rich lipoprotein metabolism [1]. Defects in triglyceride (TG) metabolism are key features in the dyslipidaemia associated with the metabolic disturbances underlying insulin resistance, metabolic syndrome, diabetes mellitus and cardiovascular disease [2]; diseases which account for high morbido-mortality worldwide. As such, knowledge of environmental and genetic factors regulating TG metabolism would help when designing strategies for improved health care for the individuals predisposed to these diseases.

In 2001, two groups identified the apolipoprotein A5 gene as being a component of the *APOA1*/*C3*/*A4* gene cluster. The gene was identified in two different settings, firstly by sequence comparison between human and mouse genomes as a putative apolipoprotein-like gene [3] and, secondly, when the molecular mechanisms and genes governing liver regeneration process in rats were being studied [4]. These studies reported that *APOA5* was exclusively expressed in the liver. Findings from animal model experiments and several association studies in humans showed that it has a major influence on circulating TG concentrations [5-8], and it has been proposed as a regulator of lipoprotein synthesis and/or secretion during the liver regeneration process [4]. The mechanism by which apo A-V would exert such an influence has not been clearly delineated. *In vitro* data suggest that it could inhibit the synthesis and/or secretion of very low density lipoprotein (VLDL) particles in the liver [9], stimulates lipoprotein lipase (LPL) activity in the presence of *APOC2* [10-13], and indirectly facilitates TG-rich lipoprotein (TRL) clearance from the plasma compartment [14-15]. Apo A-V, in contrast to other apolipoproteins, is found at very low levels in the circulation [16] and is distributed in TRL and HDL particles [16]. *APOA5* expression in animal models correlates inversely with TG concentrations while, in the majority of the studies in humans, circulating apo A-V concentrations correlate positively with TG concentrations [17-19] and, as well, with *APOB* in post-prandial chylomicrons (CM) and VLDL particles [20]. However, in view of its low concentrations, it is a matter of debate whether it can play a major role in determining the concentrations of circulating TRL.

We hypothesize that, regardless of whether its function is the intracellular control of TRL synthesis or the modulation of TG hydrolysis and lipoprotein clearance in circulation or both, it should also be expressed in the intestine where TRL are also secreted but where, to-date, apo A-V has not been identified.

3. Material and methods

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3.1. Samples

TC-7 cell culture

Human colon carcinoma TC-7 cells (subclone of CaCo-2 Cells) were purchased from Celltec (Barcelona, Spain). Cells were routinely grown in 75 cm^2 plastic flasks to a density of 1.25 x 104 cell/cm^2 and cultured in Dulbeco's modified Eagle's medium supplemented with 20% (v/v) heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37ºC in a humidified atmosphere containing 5% CO_2. All items were purchased from Invitrogen (Carlsbad, USA). When approximately 80% confluent (4-5 days after seeding) cells were trypsinised, collected and redistributed for the cell-culture experiments. The RNA experiments were performed in cells cultured (as described below) in 6-well culture dishes (Nunc, Wiesbaden, Germany). For the Western blot experiments, cells were grown in 10 cm^2 culture dishes. In all experiments, after reaching confluence (6-7 days after seeding), cells were grown for an additional 21 days for full differentiation. The culture medium was replenished three times per week.

RNA PolyA+

Human poly A+ RNA samples from liver, small intestine, duodenum, jejunum, ileum, ileocaecum and colon were obtained from BD Biosciences Clontech (Franklin Lakes, USA).

Pigs

Female pigs were fed semi-synthetic diet formulated to contain a very low level of fat and were slaughtered when an average weight of 100 kg was reached. Samples from liver and neck subcutaneous fat tissues were taken at slaughter, immediately frozen in liquid nitrogen, and then stored at -75°C until analysis. Experimental procedures were approved by the ethics committee of *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA). All procedures were within the guidelines of the IRTA, which are based on the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals.

3.2. Preparation of FA sodium salt and FA-BSA complex for cell culture experiments

The long-chain fatty acids studied were palmitic acid (PA, C16:0), oleic acid (OA, C18:1), linoleic acid (LA, C18:3), arachidonic acid (AA, C20:4), eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6). All were purchased from Sigma (St. Quentin Fallavier, France) and were subsequently prepared for the experiments according to the method of Wu et al [21], with some modifications. Briefly, 10 mg of FA were mixed with 0.5 ml ethanol (Merck, Darmstadt, Germany) and 5M

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NaOH (Fluka, St. Quentin Fallavier, France) in a volume adjusted so that the ratio of FA to NaOH was 1:1. The mixture was dried under nitrogen and then dissolved in 2 ml of sterile water ("stock solutions" of FA). To avoid FA oxidation, 1 µM butylated hydroxytoluene (Sigma, St. Quentin Fallavier, France) was added to the stock solutions of FA. When required, the stock solutions of FA were complexed to 5 mM of bovine serum albumin (Sigma, St. Quentin Fallavier, France) in a volume adjusted so that the ratio FA to BSA was 3:1. These FA-BSA solutions were sterile-filtered (0.22 µm) and used fresh.

Butyrate, the short-chain fatty acid studied, was purchased as an anhydrous sodium salt from Sigma (St. Quentin Fallavier, France). The stock solution was prepared at a concentration of 180 mM in sterile water, sterilized through a 0.22 µm filter and used fresh. Stock solutions were diluted with FBS-free culture medium and used fresh.

3.3. Effects of fatty acids and PPARα agonist on mRNA expression

Effect of fatty acids on gene expression

TC-7 enterocytes were incubated for 24h in FBS-free medium with a wide range of concentrations of FA-BSA (50,100, 200 and 300 µM) and with sodium butyrate at 2 and 5 mM. After incubation, the culture medium was collected and stored at -80ºC for subsequent analyses. The cells were used for total RNA and protein extraction. Cells incubated with vehicle alone were designated as control.

Effect of PPARα agonist on gene expression

TC-7 enterocytes were incubated with 100µM PPARα agonist, the synthetic fibrate Wy 14,643 (Calbiochem, Darmstadt, Germany), in a serum-free medium. After 24h incubation, the culture medium was collected and stored at -80ºC for the subsequent analyses. The cells were used for total RNA extraction. Cells incubated with vehicle alone (DMSO; Sigma, St. Quentin Fallavier, France) were designated as control.

3.4. Cytotoxicity

The cytotoxic effects of the FAs studied and the synthetic fibrate were measured as the amount of lactate dehydrogenase (LDH) released into the culture medium. LDH was measured in an automated analyzer (Cobas-Mira; Roche, Switzerland) using an enzymatic method (Roche Molecular Biochemicals). Cytotoxicity was defined as the LDH release significantly greater than that measured in cells not treated with each FA or the PPARα agonist. LDH was expressed as units per litre (U/L). Alterations in cell morphology were evaluated using phase contrast microscopy.

3.5. RNA extraction and real-time quantitative PCR

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Total RNA was extracted from cultured cells and pig liver tissue using the ABI PRISM 6100 Nucleic Acid PrepStation extraction system (Applied Biosystems; Foster City, USA) according to the manufacturer's instructions. Total RNA was extracted from pig adipose tissue using 1.5 ml TRIZOL (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

The purity and concentration of RNA was estimated by the 260 / 280 nm absorbance ratio. RNA (1 µg) was retrotranscribed into cDNA using random hexamers with the reverse transcriptase SuperScript II (Invitrogen, Carlsbad, USA) following the manufacturer's instructions and using the PE Biosystems 2400 thermocycler (Foster City, USA).

The mRNA expression of the genes under study was semi-quantified by real time PCR using the ABI Prism 5700 Sequence Detector System (Applied Biosystems, Foster City, USA), combined with primers and Taqman® probes obtained from Applied Biosystems as pre-designed and validated "Assays-on-Demand" products. The GAPDH gene was used as endogenous control and the expression levels were calculated using the ΔΔct method (ABI Prism 7700 Sequence Detection System; user bulletin nº2. Revision A. Foster City (USA): Applied Biosystems, 1997).

3.6. Protein extraction and Western Blot of apo A-V

TC-7 enterocytes were cultured as described above and were incubated with the FA-BSA complex (EPA and DHA 200µM) for 24h after which whole cell lysates (WL) were obtained by repeated freezing and thawing in homogenisation buffer containing PBS pH 7.4 and complete mini protease inhibitors (Roche, Manheim, Germany). Samples were stored at -80ºC for subsequent assay. Total protein was measured using the Bradford method [22]. Electrophoresis and blotting was performed using NUPAGE® Protein Analysis System (Invitrogen, Carlsbad, USA). Blotting membrane was blocked with 4% ECL Advance Blocking Reagent (Amersham Biosciences, Bucks, UK) and then incubated with the anti-apo A-V antibody at 1:2500 dilution (Novus Biologicals, Littleton, USA). Antigen-antibody complexes were detected by incubating the membrane with polyclonal goat anti-mouse Immunoglobulins/HRP (1:20000, P0447, Dako, Glostrup, Denmark). Signals were detected with ECL advanced reagent mixture and the bands were visualized on film by autoradiography. Banding patterns were captured using a Versadoc 4000 imaging system and analysed using the Quantity One software (Bio-Rad Laboratories, Hercules, California, USA). Actin was used to normalise the results obtained in each sample. The protein sizes were confirmed by comparison with molecular weight standards (Invitrogen Ltd, Paisley, UK).

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3.7. Statistical analysis

A Pearson Correlation was calculated examining the relationship between variables. The expression of *APOA5* gene in TC-7 cells incubated with long-chain fatty acids was statistically evaluated using the ANOVA test to compare the effects of the different groups of FA (saturated, monounsaturated, polyunsaturated ω-3 and ω-6). Data from the incubation of the TC-7 cells with different concentrations of short-chain butyrate were from a one-off experiment. Data from the incubation of the TC-7 cells with different concentrations of the PPARα agonist were statistically evaluated using ANOVA. A p value of <0.05 was considered significant.

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4. Results

4.1. APOA5 in TC-7 cells and human intestine

Using quantitative real time RT-PCR with the GAPDH gene as the endogenous control, we evaluated *APOA5, APOB, MTP* and *APOC3* gene expression in human poly A+ RNA from samples of duodenum, jejunum, ileum, ileocaecum and colon. We took the highest expression levels observed for each gene in the intestinal segment as the reference value. We were able to detect *APOA5* expression in the concluding cycles of amplification (which indicates a low level of expression) and found that *APOA5* is expressed mainly in duodenum and colon. Conversely, *APOB* was found to be expressed mainly in duodenum, jejunum and ileocaecum with *MTP* in duodenum and jejunum, and *APOC3* in jejunum (Figure 1). We found an inverse correlation although non-significant between *APOA5* and *APOC3* gene expression in every human intestinal section analysed (r=-0.555, p=0.166).

A Western blot was optimised to detect apo A-V protein levels in whole lysates obtained from TC-7 cells, from hepatic HepG2 cells used as positive control, and from monocyte THP-1 cells used as a negative control. These results confirmed those obtained from the gene expression assays i.e. the existence of apo A-V in intestinal TC-7 cells at approximately one third of that found in liver cells (Figure 2), and the absence of apo A-V in monocytes.

4.2. Incubation of TC-7 cells with fatty acids

The positive results obtained in human intestine were confirmed in cultured cells and, in view of the low expression levels, we tested whether its expression was modifiable by factors known to affect lipid metabolism, such as diet and certain pharmacological agents. Initially, we incubated TC-7 cells for 24h with long-chain fatty acids (palmitic acid as saturated FA, oleic acid as monounsaturated FA, eicosapentaenoic acid and decosahexaenoic as polyunsaturated ω-3 FA, linoleic and arachidonic acids as polyunsaturated ω-6 FA) at increasing concentrations (from 50 to 300 μM). Relative to control cells (i.e. those not incubated with any fatty acid) and with the exception of palmitic acid, the level of *APOA5* expression was decreased by fatty acid addition in a weak dose-dependent manner, and the effect of saturated fatty acid on *APOA5* gene expression was significantly different from that caused by PUFA ω-3 and ω-6 fatty acids (p=0.012 and p=0.004, respectively) (Figure 3).

Based on its known role in regulating TG metabolism, we tested the effect of the short-chain fatty acid butyrate on intestinal *APOA5* gene expression by incubating TC-7 cells with butyrate at 2 and 5 mM for 24 hours. The 5mM butyrate incubation induced almost

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4-fold increase in *APOA5* gene expression. The measured *APOB* mRNA levels were found to be down-regulated by increasing concentrations of butyrate (1.27 vs. 0.72, $2^{-\Delta\Delta ct}$ at 2 and 5 mM respectively, relative to expression levels in control cells) (Figure 4).

4.3. Incubation with PPARα agonist

Fibrates are the treatment-of-choice for lowering TG concentrations. To test whether intestinal *APOA5* gene expression responds to fibrates, we incubated TC-7 cells with the synthetic fibrate Wy 14,643 at 100 μM for 24h. Incubation with Wy 14,643 increased *APOA5* expression by 60% relative to control cells ($p=0.008$). Under the same conditions *APOB* expression was decreased by 50% ($p<0.0001$), *MTP* was decreased by 30% ($p=0.002$) and *APOC3* also decreased by 45% ($p=0.004$) (Figure 5).

4.4. Animal model

In humans, *APOA5* is expressed exclusively in the liver; the main site of lipid synthesis. We used the porcine model because the adipose tissue, not the liver, is the main site of lipid synthesis [23]. We hypothesised that *APOA5* would be present in the adipose tissue but not in the liver.

Since *APOA5* has, as-yet, not been described in the pig, we initially searched for high-homology sequence regions in *APOA5* mRNA between species (human and mouse) on the assumption that these could be used as pre-designed and validated TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, USA) for human and mice *APOA5* and, as such, could be applied in the porcine model. In line with this proposal, we were able to detect *APOA5* expression in the adipose tissue, but not in the liver, of the pig.

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5. Discussion

We hypothesised that apo A-V is not exclusively expressed in the liver. Our present findings confirm that apo A-V is also expressed in human intestine and in TC-7 cells. Moreover, its expression is modulated by fatty acids and PPARα agonist.

5.1. APOA5 expression in human intestine

We found that *APOA5* is expressed at low levels in the TC-7 intestinal cells, and also in human duodenum and colon. This implies that all genes of the A1-C3-A4-A5 gene cluster are transcribed in the intestines [24]. Apo A-I and apo C-III participate in HDL and TG metabolism, whereas apo A-IV, the amino acid sequence of which bears strong similarity to that of apo A-V, is involved in intestinal lipid absorption, mainly in duodenum and jejunum [25].

Whether such low intestinal expression of *APOA5* can be functional, and what could be its function, remains an unanswered question.

APOA5 is the one component of the *APOA1/C3/A4/A5* gene cluster that is not transcriptionally regulated by the *APOC3* enhancer and which may explain, in part, the low expression levels. Also, we believe that a certain difference in apo A-V concentrations between intestine and liver, in favour of the liver, would be expected considering that the liver is the main organ in the body that mobilises endogenous lipids and controls lipid homeostasis [26]. Indeed, Western blot analyses in TC-7 cells indicate that, despite low transcription rates, apo A-V protein concentrations amount to approximately one third that of liver cells.

We are aware that the mere presence of apoA-V in the intestine does not indicate its potential function i.e. control of TRL synthesis and secretion, or TG hydrolysis and remnant particle clearance. For example, apoA-V may be produced in the intestine to be secreted with HDL for subsequent transfer to TG-rich particles. Also, as has been proposed by others [27] it may be secreted within TRL derived from the intestine in order to stimulate TG hydrolysis, like an antagonist to the intestinal apoC-III. This is in accord with the strong inverse correlation between the levels of *APOA5* and *APOC3* gene expression we observed in the different human small intestine sections. Another function could be to regulate the assembly and secretion of intestinal lipoproteins, replete with dietary lipids. In support of this function was our observation that its expression is affected by FA and, as such, favours the description of its role in TG metabolism in which it is modulated by dietary factors [28].

However, our findings support the notion that *APOA5* is expressed in those tissues which synthesise and secrete lipids i.e. the liver as well as the intestine. Conversely, in the pig, in which lipid synthesis and secretion take place mainly in the adipose tissue

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rather than the liver [29], we found that *APOA5* is expressed in adipose tissue, and not in the liver. This is very much in line with data reported from another animal model, the chicken. In this model it is the kidney which is a very active site of lipoprotein synthesis; immunoreactive apoA-V being identified in various chicken tissues and with the highest expression being in the kidney [15].

5.2. APOA5 responds to fatty acids and PPARα agonist

To seek further evidence of its possible functionality, we assessed the responsiveness of expression of intestinal apo A-V to agents that would affect its low expression i.e. agents such as dietary fatty acids or a PPARα agonist.

We studied the modulatory effect of long-chain fatty acids on intestinal *APOA5* expression in the CaCo2 / TC-7 cell model based on the observation that long-chain fatty acids are specific ligands for PPAR nuclear transcription factors, to which *APOA5* is responsive [30, 31]. We found that monounsaturated and polyunsaturated fatty acids tended to decrease intestinal *APOA5* expression while saturated fatty acids tended to increase the expression. This would imply that apo A-V would downregulate intestinal lipid mobilisation in a situation of high intake of saturated fat.

The site for the formation of short-chain fatty acids from dietary soluble fibre, which have a lowering effect on triglycerides and cholesterol [32], is the colon. In view of the relatively high *APOA5* expression in the colon, we evaluated the modulatory effect of short-chain fatty acids (i.e. butyrate) on intestinal *APOA5* expression. Butyrate decreases hepatic and intestinal protein expression of *APOB* and *MTP* [33] and which, in our cell culture experiments, induced a 4-fold increase in APOA5 gene expression. This is consistent with its potential role of inhibiting TRL production.

And finally, as has been described in human hepatocytes [30], we have found that *APOA5* gene expression in intestinal TC-7 cells is also upregulated by a PPARα agonist. This result is of considerable clinical interest because fibrates, which are PPARα agonists, are the best therapeutic option to reduce TG and to increase HDL-c [34]. Activated PPARα heterodimerises with other nuclear transcription factors such as retinoid X receptor (RXR) and up-regulates the *LPL*, *APOA1* and *APOA5* genes and down-regulates *APOC3*. The outcome is a decrease in plasma TG concentrations due to a combined action of reduced triglyceride synthesis as well as increased catabolism.

In summary, our results demonstrate that *APOA5* is not exclusively synthesised in the liver, but also in the intestine, where is expressed at low levels and is responsive to fatty acids and fibrates.

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Acknowledgements

The study was supported by CIBER (*Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas*) which is an initiative of Spanish Ministry of Health via the ISCIII (*Instituto de Salud Carlos III*).

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Figure legends

Figure 1: Human polyA+ RNA from the different sections of intestine was obtained from BD Biosciences Clontech to perform the real time RT-PCR for *APOA5* (panel A), *MTP* (panel B), *APOC3* (panel C) and *APOB* (panel D) to determine gene expression levels.

Figure 2: Cell lysates from intestinal TC-7 cells, hepatic HepG2 cells (as positive control) and from monocytes THP-1 (as negative control) were used to detect apo A-V protein levels by Western blot. Intestinal apo A-V levels are approximately one third of that in hepatocytes.

Figure 3: Total RNA from intestinal TC-7 cells incubated with different long-chain fatty acids [palmitic acid (PA), oleic acid (OA), linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and decosahexaenoic (DHA)] at different concentrations [50μM (black), 100μM (white), 200μM (grey) and 300μM (striped)] over 24h was isolated to determine *APOA5* gene expression levels by RT-PCR and compared to control cells (not incubated with any FA). * *APOA5* expression significantly different among saturated FA and PUFA ω-3 (p=0.012); ** *APOA5* expression significantly different among saturated FA and PUFA ω-6 (p=0.004).

Figure 4: *APOA5* (panel A) and *APOB* (panel B) gene expression dose response (2 and 5 mM) to short-chain fatty acid butyrate incubation in intestinal TC-7 cells for 24h, analysed by RT-PCR, and compared to control cells (not incubated with butyrate acid).

Figure 5: Effects of 100 μM Wy 14,643 (PPARα agonist) incubated over 24h in TC-7 cells on *APOA5*, *APOB*, *MTP* and *APOC3* gene expression analysed by RT-PCR, and compared to control cells (not incubated with Wy 14,643). Expression level of *APOA5* was significantly upregulated by the agonist (p=0.008). Conversely, *APOB*, *MTP* and *APOC3* expression were downregulated (p<0.0001, p=0.002 and p=0.004, respectively).

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Figure 1

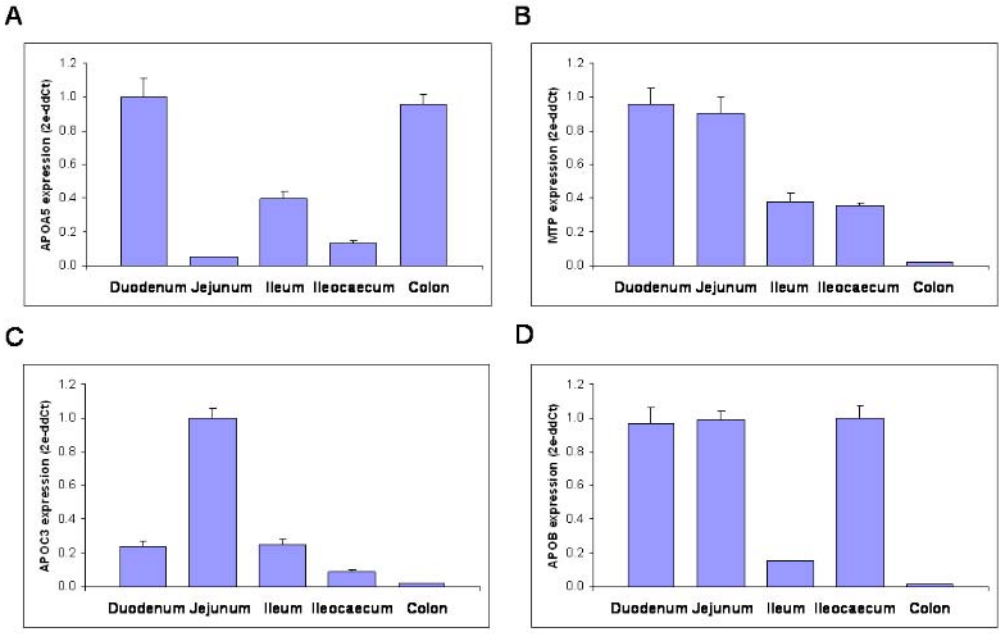

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Figure 2

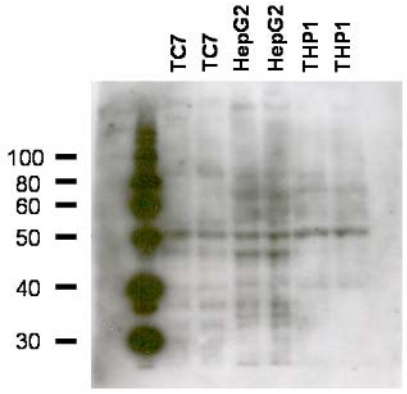

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Figure 3

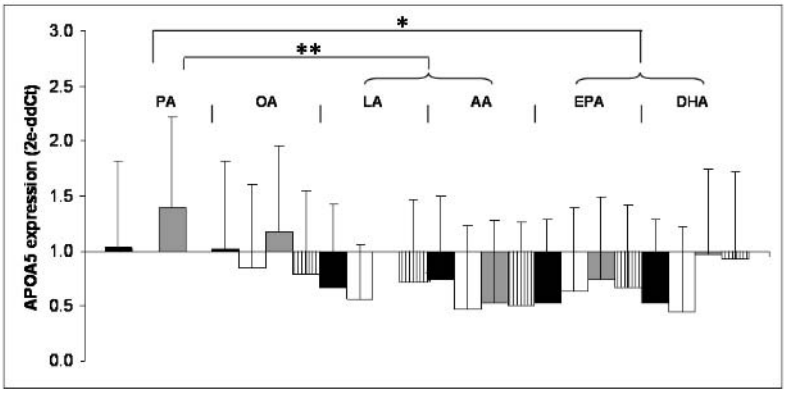

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Figure 4

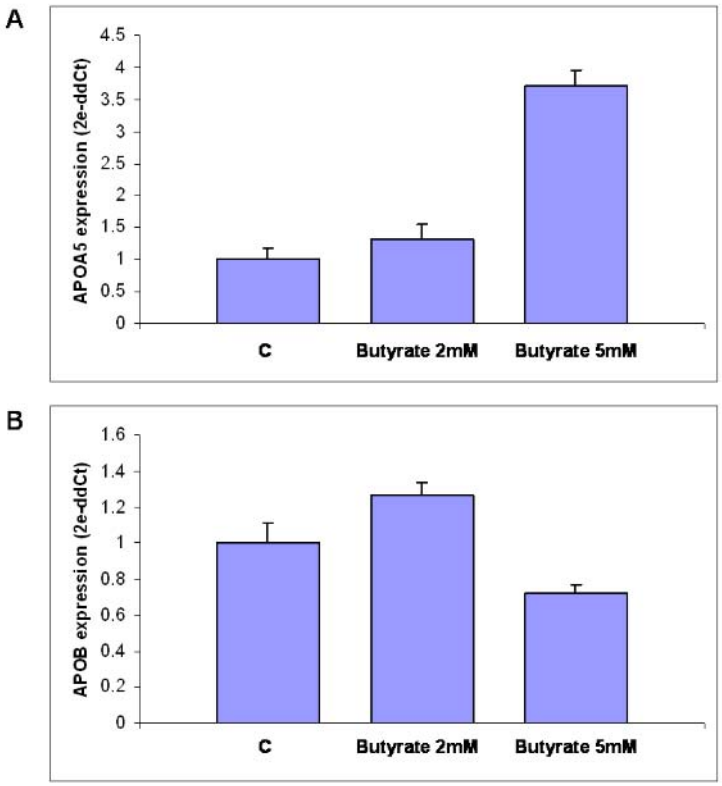

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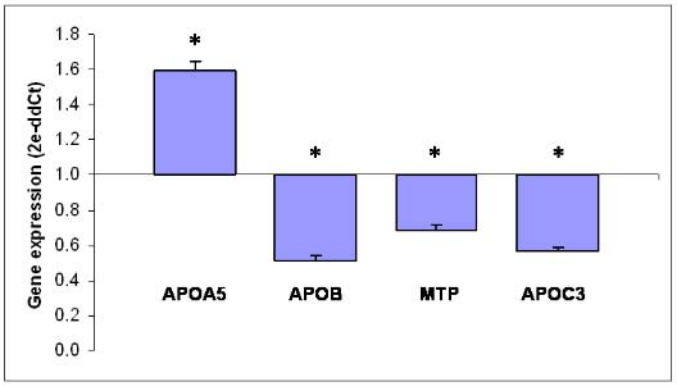
Figure 5



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Conclusions

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Conclusions

- The strong and consistent association between APOA5 and the levels of TG demonstrated in several published studies can be explained by an increase in the content of VLDL in circulation.

- The genetic variant -1131T>C of the APOA5 gene is associated with higher concentrations of circulating α-tocopherol. The metabolism of vitamin E occurs in parallel to that of the TG-rich lipoproteins and, as such, we postulate that the effect of APOA5 on the levels of vitamin E is probable due to the effect of the variant of the gene on VLDL.

- The effect of APOA5 on the levels of vitamin E is not accompanied by differences in the oxidative status of the subjects because, although the individuals have high levels of oxidative substrate (TG), they also have a greater quantity of antioxidant (vitamin E).

- The -1131T>C variant predisposes to hyperlipidemia in patients with HIV treated with protease inhibitors. The differences with respect to the atherogenic profile between genotypes are not reflected in the sub-clinical arteriosclerosis measured over a period of two years of follow-up.

- The diet rich in saturated fatty acids decrease the hepatic expression of the APOA5 gene in mice deficient in APOE, and the addition of cholesterol reverses this effect. The hepatic expression of APOA5 in this animal model correlates with the arteriosclerosis and with inflammation.

- The APOA5 in APOE deficient mice has a minimum effect on the concentrations of plasma TG, probable explained by the defect in the secretion route of TG in the liver in this animal model.

- The hepatic expression of APOA5 increases with age in APOE deficient mice, and is in the same direction as the relationship between APOA5 and the grade of arteriosclerosis and inflammation, given that these processes worsen with age in these animals.

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- APOA5 is not expressed solely in the liver and, although it is expressed in the human intestine at low levels, its expression can be modified by the intake of fatty acids and the use of fibrates. This suggests that it can play a role in the regulation of lipid metabolism in response to dietary and pharmacological interventions.

- The *in vivo* expression of APOA5 in the intestine correlates inversely with that of APOC3.

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