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Serum lipoprotein functionality and cellular lipid transporters: focus on their possible role on cardiovascular risk modulation

Coordinatore:
Chiar.mo Prof. Marco Mor

Tutore:
Chiar.ma Prof.ssa Elda Favari

Dottorando: Annalisa Ronca

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Abstract

Cardiovascular disease (CVD) is a leading cause of death with over 17 million deaths annually according to the World Health Organization. Among the major risk factors we find LDL that, accumulating in macrophages, lay the foundations of atherogenesis. This pro-atherogenic mechanism is opposed by the reverse transport of cholesterol (RCT), thanks in particular to its first step, the efflux of cellular cholesterol, mediated by the interaction of serum HDL with the different cell transporters. Studies show that HDL quality, beyond than quantity, is a better predictor of CVD risk.

In this context, evaluating HDL functionality through cholesterol efflux capacity (CEC), would represent a better approach to stratify CVD risk, independently of classical risk factors. CEC is therefore an anti-atherogenic parameter, that has been demonstrated to be inverselly correlated with CVD risk.

The amount of cholesterol inside cells is determined not only by the quantity that comes out of cells, but also by the quantity that enters. For this reason, a pro-atherogenic parameter has been identified to evaluate the ability of a serum to transfer cholesterol to the cell, called cholesterol loading capacity (CLC).

Therefore, in the first project of this thesis we wanted to analyze CLC in patients with high levels of Lp(a) and at high risk of coronary heart disease (CAD) undergoing apheresis. Lp (a) is a lipoprotein more available to oxidation than LDL, thus representing a CVD risk factor. Among the gene determinants of Lp(a), to remember is a structure called KIV2, which determines by varying in number of copies, not only the quantity but also the size of the lipoprotein.

Our study demostrated that particles of smaller size are associated with a higher number of Lp(a), while larger particles are associated with a smaller number. In addition larger Lp(a) particles determine a lower CLC and consequently a lower CVD risk.

Not only do gene alterations represent a factor capable of influencing CVD risk, but metabolism would also represent a possible cause capable of influencing this risk.

For this reason the aim the second project was to define a possible cause of the different CVD risk in patients with metabolic NAFLD compared to patients with NAFLD on a genetic basis. This disorder, caused by an excessive accumulation of TG in the liver, is actually a multifactorial pathology.

In this study we observed how patients with the metabolic form were showing a decrease of serum CEC and an increase of serum CLC, which was not found in patients with genetic form. The levels of fat are not decisive, in fact, despite the same amount of fat, patients affected by the metabolic form have a lower CEC and a higher CLC than the genetic group, which had no different parameters compared to control subjects.

Metabolic alterations would therefore appear to be the necessary cause of the increased CVD risk that afflicts patients with metabolic-based NAFLD, and the presence of metabolic syndrome would further aggravate the clinical picture.
If on the one hand genetic and metabolic alterations are capable of influencing CVD risk, on the other hand an approach able to modify this risk would be represented by the diet.
In the third project of this thesis we wanted to evaluate if two different dietary approaches, namely the Mediterranean diet (MD) and the vegetarian diet (VD), were able to affect CEC of the HDLs in healthy patients with low or medium CVD risk. VD was observed to reduce both Total-CEC and ABCA1-mediated CEC, while passive diffusion-mediated CEC was unchanged compared to the MD diet. The benefits of MD may be due to the lipid-lowering effects as well as the impact on some HDL functions, such as the antioxidant, vasodilatatory capacity and the ability to promote cholesterol efflux .
In the last project, we evaluated a possible mechanism of action of a plasma CVD risk biomarker, namely anti-APO-AI antibodies. Anti-Apo-AI IgG are now known to be associated with various pathologies not only on inflammatory basis but also on immune ones. In this study we observed how these autoantibodies are able to maintain unchanged the expression of an important lipid metabolism regulator gene, namely SREBP-2, while increasing the expression of another TG metabolism gene, namely SREBP-1. Conversely, the expression of other genes, such as GPAT1 and FASN, which are also involved in metabolism, would be reduced. An induction of the inflammatory stimulus via TLR-2 would also occur in the presence of these antibodies.
Therefore, Anti-Apo-AI IgG would appear to be capable of inducing lipid accumulation at the cellular level through this gene regulation, potentially responsible for the onset of diseases such as NAFLD.

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Introduction

1. Atherosclerosis: general aspects and risk factors

Atherosclerotic cardiovascular diseases (ASCVD) are the leading cause of morbidity and mortality worldwide with over 3.9 million deaths per year in Europe (Sanz 2020).

Atherosclerosis is a chronic inflammatory pathology, localized to the arterial wall, especially at the level of the branch points, where the laminar flow is disturbed (Moore 2011). This inflammatory process is caused by the accumulation of Apo B-lipoproteins and fibrotic material in large and medium sized arteries (Lusis 2000).

These lipoproteins undergo oxidation, cleavage and aggregation processes that make these particles pro-inflammatory and determine the activation of the overlying endothelium (Moore 2013). This triggers an immune response with the recruitment at the subendothelial level of monocytes, which differentiate into macrophages, that engorged with these lipoproteins, are transformed into "foam cells". The uptake of these modified lipids by macrophages causes the endothelial cells (EC) to express adhesion molecule and release chemokines; together these events induce the infiltration of intimate immune cells. The final result is the formation of early lesions (Weber 2011). Next, apoptotic cells and debris accumulate to form a necrotic nucleus. A fibrous cap, consisting of smooth muscle cells (SMCs) and collagen, is formed around this nucleus (Lusis 2000; Ylä-Herttuala 2011). The structure, containing a necrotic central nucleus and a fibrous cap, forms the atherosclerotic plaque or atheroma, the typical lesion of atherosclerosis (Robbins S.L. e Cotran R.S. 2006).

Macrophages, that accumulate inside the atherosclerotique plaque, have a reduced migration capacity. This implies a failure in the resolution of the inflammatory process and the progression of the lesion (Randolph 2008). Nevertheless, the recruitment of other monocytes and leukocytes to the artery is important to promote the regression of the pathology and induce the resolution of inflammatory processes (Nathan 2010). Studies on regression models of atherosclerosis show that macrophage recruitment can be reduced by acting on the steps leading to atherosclerosis in the plaque (Feig, Parathath 2011; Feig, Rong 2011). This allowed identification of new targets in the plaque, as both the phenotype and the quantity of macrophages determine the atheromatous inflammatory state.

The typical consequence of the atheroma formation is the protrusion of the newly formed mass towards the lumen of the vessel, thus affecting blood flow; however, the most relevant clinical condition is the sudden occlusion of the vessel due to the formation of a thrombus or a clot, which leads to heart attack or stroke (Robbins S.L. e Cotran R.S. 2006). Another clinical condition is the rupture of the fibrous cap, occurring in the vulnerable and unstable plates, where the fibrous cap is thinner. A thin fibrous cap is more prone to ruptures, followed by thrombotic events. A thick cap is

more stable and does not allow the contact between the core of the plaque, rich in necrotic factors, and the blood flow.
Due to the late clinical manifestations of this disorder, it is important to identify, correct and control risk factors.
Studies such as the Framingham Heart Study and the Multiple Risk Factor Interventional Trial (MRFIT), have allowed the identification of risk factors correlated with the development of atherosclerosis and cardiovascular disease (CVD). There are two distinct categories of risk factors for the onset of atherosclerosis: the modifiable, that correctable through pharmacological or behavioral interventions, and the non-modifiable ones.
Dyslipidemia, and in particular hypercholesterolemia, is an important modifiable risk factor, representing a sufficient condition for the formation of lesions. In particular, elevated total cholesterol levels, reduced HDL cholesterol levels (HDL-C) and elevated LDL cholesterol levels (LDL-C), as well as triglycerides (TG) represent an atherogenic profile (Kumar, Abbas 2011). Hypertension, consisting of blood pressure values above 140/90 mmHg, is another cardiovascular risk factor that determines the onset of typical cardiovascular diseases such as stroke, myocardial infarctions and endothelial lesions (Kumar, Abbas 2011). Smoking increases coronary risk, at least 3 times compared to non-smokers (Grundy 2004). Diabetes, another independent risk factor, is associated with an increased risk of myocardial infarction, stroke, and peripheral arterial disease. The risk is especially evident in young and female patients (Almdal 2004; Zavaroni 1989).
Overweight and obesity, in particular an increase in body mass index (BMI) and waist circumference, are associated with cardiovascular risk (Gami 2007; Wilson 2008).
Advanced age, especially for men where the risk increases over 45 years, whereas for women over 55, is linked to the increase in coronary risk (Robbins S.L. e Cotran R.S. 2006). This shift in cardiovascular risk towards a higher age in women is due to the protective role of estrogen, which disappears during menopause (Kumar, Abbas 2011).
Sedentary lifestyle as well as incorrect eating habits have an impact on the lipid and lipoprotein profile. Physical activity allows for a decrease in LDL-C levels and an increase in HDL-C levels with an atheroprotective effect (Hu 2007; Imes 2014; Wang 2017).
Familiarity with cardiovascular disease is also a risk factor (Imes 2014).
Additional risk factors related to cardiovascular risk have recently been identified.
C-reactive protein (CRP), produced by the liver during a state of acute inflammation, after stimulation by some pro-inflammatory cytokines, is consider a risk factor. Since atherosclerosis is an inflammatory pathology, CRP could be a good marker, as this protein appears to be able to promote atherosclerosis, increasing the phagocytosis and chemotaxis processes of monocyte-macrophage

populations (Mozos 2017). However, it must be said that this process would seem to be non-specific, because levels of this protein can be influenced by other factors such as age, sex and physical state. Despite this, an association has been determined between increased CRP levels and cardiovascular risk in healthy patients (Corrado 2007).
Moreover, high levels of white blood cells, erythrocyte sedimentation rate (ESR), interleukin-6 (IL-6), interleukin-18 (IL-18), tumor necrosis factor (TNF-α) and fibrinogen, all inflammatory markers, may be linked to cardiovascular risk (Mozos 2017).
Another factor linked to atherosclerosis seems to be myeloperoxidase (MPO). It is a heme peroxidase, an enzymatic source of bioactive lipids and eicosanoids, capable of generating atherogenic forms of both low and high density lipoproteins, thus influencing the state of the arterial wall (Nicholls 2008). Clinical studies show an increased CVD risk in the presence of high levels of MPO, which might act as a catalyst for LDL oxidation processes (Delporte 2013).

2. Cholesterol: structure, synthesis and biological function

Cholesterol is a lipid that plays an essential role in the composition and functioning of biological membranes. It has the task of maintaining cellular homeostasis, regulating communication between cells and the extracellular environment, as well as modulating the structure and function of membrane proteins. It is also a necessary element for the synthesis of steroid hormones, vitamin D and bile acids (Ding 2019; Plat 2019; Rahmati-Ahmadabad 2019).
The first to identify this molecule inside gallstones was François Poulletier de la Salle in 1769, but it was named as cholesterol only in 1815 by Chevreul. Between 1908-1913, cholesterol was recognized be involved in the development of vascular diseases such as atherosclerosis, by studies of changes in the vasculature of rabbits fed fatty and high cholesterol foods (Kuijpers 2021; Sedaghat and Grundy 1980).
Structurally cholesterol is an amphipathic molecule, composed of 27 carbon atoms (Figure 1) It is characterized by a hydroxyl group on the C-3 atom, representing the polar head, and a steroid nucleus (characteristic of steroid hormones) with two groups methyl and hydrocarbon chain on the C-17 atom to form the non-polar hydrocarbon body (Craig 2021).

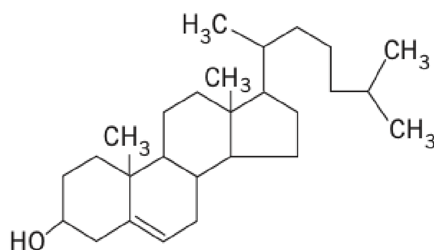


Figure 1. Cholesterol Structure (Crook 2012)

Due to its amphipathic properties cholesterol can be incorporated into the phospholipid bilayers of biological membranes. In the phospholipid bilayer, cholesterol orients its hydroxyl group towards the polar heads of the phospholipids, in contact with the aqueous extracellular environment. The steroid nucleus, which has a relatively rigid planar structure, contacts the proximal -CH2 groups of the fatty acid chains. Thanks to this spatial arrangement, cholesterol limits the freedom of movement of the aliphatic chains of phospholipids and therefore reduces the fluidity of the biological membrane, thus increasing its mechanical stability and giving it greater structural integrity.

The structure of cholesterol involves a complex synthesis pathway involving more than 25 enzymes. The precursor, from which its carbon atoms derive, is acetate. The isoprene units, necessary intermediates of the acetate pathway leading to the synthesis of cholesterol, are also precursors of other natural lipids and share the same biosynthetic reactions (Nelson, Cox 2008).

The summary of cholesterol biosynthesis includes the following steps (Nelson, Cox 2008): 1) the condensation of three acetate units and subsequent formation of an intermediate with six carbon atoms, mevalonate. The rate-limiting enzyme is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), that is regulated with a negative feedback mechanism by the IC concentration; 2) conversion of mevalonate into activated isoprene units; 3) polymerization of isoprene units with five carbon atoms to lead to the formation of squalene, a linear compound characterized by the presence of 30 carbon atoms; 4) cyclization of squalene to form the four-ringed steroid nucleus, from which cholesterol is obtained through a series of chemical modifications (oxidation, removal or migration of methyl groups).

This pathway, called the "mevalonate pathway", also leads to the production of lipid, farnesyl pyrophosphate, which is necessary for the anchorage of membrane proteins, and the production of the heme prosthetic group for cytochromes, dolichol and ubiquinone.

An important transcription factor in de novo synthesis of cholesterol is the sterol regulatory element-binding protein 2 (SREBP-2) (Weber 2004). Two situations can occur depending on whether intracellular (IC) cholesterol levels are elevated or reduced. In the first case, its precursor creates a complex with the insulin-induced gene and the SREBP cleavage activating protein (SCAP) and is

retained in the ER membrane. In the second case SCAP accompanies the precursor into the Golgi, where S1P and S2P, proteases sensitive to steroids, cleave an N-terminal fragment, which is then translocated into the nuclei to activate the transcription of the LDL receptor (LDL-R) and various genes involved in de novo synthesis of cholesterol. Once activated, SREBP-2 binds to the steroid response element (SRE), at the promoter level of cholesterogenic genes, and subsequently activates their gene transcription (Chiang 2014).

Interestingly, in vertebrates most of the cholesterol (about 80%) is produced by the liver, and only a small portion is incorporated into the membranes of the hepatocytes, as the greater part is exported in one of three possible forms: bile cholesterol, bile acids or cholesterol esters.

About 20% of total cholesterol is introduced with foods, especially of animal origin, such as dairy products. It is then absorbed by the intestine and transported to the liver where it is processed and incorporated into the lipoproteins, as, due to its structural characteristics, cholesterol cannot mix with water, and is therefore packaged together with the apoproteins (proteins) to be transported through the bloodstream in the form of lipoprotein (Crook 2013).

3. Lipoproteins

Lipoproteins are spherical macromolecular complexes consisting of various proteins and several hundred hydrophobic and amphipathic organic molecules (Daniels 2009). As shown in Figure 2, lipoproteins have a structure characterized by a hydrophobic inner core, composed of non-polar lipids, such as cholesterol esters and triglycerides, and a hydrophilic outer membrane layer, consisting of apoproteins, phospholipids and free cholesterol. These aggregates collect and transport lipids, such as free cholesterol, which are inherently insoluble in water, in the blood (Chiang 2014).

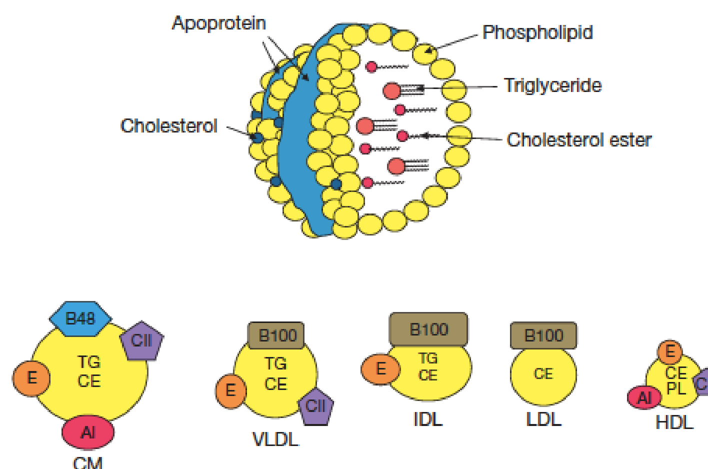


Figure 2. Lipoprotein Structure (Chiang 2014)

Lipoproteins differ in terms of density, lipid composition and nature of the apolipoprotein portion. Apolipoproteins determine the metabolic fate of lipoprotein particles by acting not only as structural elements that stabilize the particles (Apo B, Apo A-I, Apo A-II), but also as membrane receptor ligands (Apo B, Apo E) and activators or inhibitors of enzymes (Apo A-I, Apo C-II, C-III) or transport systems (Apo A-IV, Apo F) (Cohen 2013).

Lipoproteins are classified according to some of their physico-chemical characteristics. In relation to their density, diameter, composition and electrophoretic mobility, lipoproteins can be classified into 7 main classes (Table 1) (Feingold 2000):

1. Chylomicrons: characterized by very low density (d<0.93 g/ml) but with a greater diameter (75-1200nm); the lower density is due to their almost exclusive content of triglycerides (90%) and a small percentage of cholesterol (3-5%). The apolipoproteins associated with chylomicrons are mainly Apo B-48, Apo E and Apo C. They are synthesized at the enterocyte level and have the function of collecting lipids (derived from food), triglycerides and cholesterol in the intestine. Being formed in the intestine, chylomicrons are released into the bloodstream and distribute lipids to tissues. They deposit triglycerides at the level of adipose tissue and muscle tissue: in the capillaries, the endothelial cells express the lipoprotein-lipase which breaks down triglycerides into fatty acids and glycerol. Chylomicrons sequester triglycerides and are converted into remnants that are captured by the liver (Mansbach and Siddiqi 2010).
2. Chylomicrons remnants: transfer triglycerides from chylomicrons to peripheral tissues results in the formation of smaller particles (30-80 nm) called remnants, richer in cholesterol than chylomicrons from which they derive (Cooper 1997).
3. Very Low-Density Lipoproteins (VLDL): are very low density lipoproteins (d = 0.93-1.006 g/ml), with a diameter of 30-80 nm, and with a protein percentage slightly increased compared to chylomicrons. VLDL are synthesized in hepatocytes and contain esters and triglycerides. They transport in the bloodstream cholesterol esters and triglycerides of endogenous origin, synthesized by the liver, muscle tissue and adipose tissue. VLDL are smaller than chylomicrons, and the insize depends on the amounts of triglycerides contained, similar to chylomicrons.

   VLDL apolipoproteins consist of Apo B-100, C-I, C-II, C-III and E, of which Apo B-100 is the main structural protein, and each VLDL particle contains a molecule of Apo B-100 (Cox and García-Palmieri 1990).
4. Intermediate-Density Lipoproteins (IDL, VLDL Remnants): have intermediate density (1.006 <d<1.019 g/ml) and a diameter of 25-35 nm. They have a reduced content of triglycerides,

but a higher quantity of esterified cholesterol. They result from the removal of triglycerides from VLDLs in muscle and fat tissue. The protein part of these lipoproteins is represented, mainly, by apolipoprotein B-100 and E (Feingold 2000).

5. Low-Density Lipoproteins (LDL): they have low density (1.019-1.063 g/ml), deriving from VLDL and IDL, with increased protein percentage and are particularly rich in cholesterol esters. LDLs vary widely in size and density. They are responsible for transporting ester cholesterol to the tissues, and they also can return to the liver. They have a single Apo B-100 on the surface that allows them to bind the receptors present on the liver, leading to their internalization (Ference 2017). Small and dense LDL particles (sdLDL) are associated with an increased cardiovascular risk, and are therefore more atherogenic for several reasons: 1) reduced affinity for LDL-R, with a greater permanence in the circulation; 2) more easily penetrate into the arterial wall, and remain trapped in the vessels due to binding with intra-arterial proteoglycans; 3) increased sensitivities to oxidation, resulting in uptake by macrophage scavenger receptors (Gerber 2017).
6. High-Density Lipoprotein (HDL): They are the smallest lipoproteins, around 5-12 nm, and based on density they are divided into HDL2 (1.063-1.120 g/cm3) and HDL3 (1.120-1.210 g/cm3) (Barter 2003). These particles are associated with apolipoproteins AI, A-II, A-IV, CI, C-II, C-III and E. The main structural protein is Apo A-I and each HDL particle can contain multiple molecules of Apo A-I (Feingold 2000). They are capable of carrying phospholipids and cholesterol esters and the protein component is 70% Apo A-I and 30% Apo A-II. The protein part constitutes 50% of HDL, while the lipid part the remaining 50%. HDLs, enriched in cholesterol and phospholipids, are a very heterogeneous family and are involved in the athero-protective process of reverse cholesterol transport (RCT). They originate in the liver and small intestine in the form of small particles rich in proteins, low in cholesterol and free of cholesterol esters. When they are enriched with free cholesterol, derived from membranes, tissues and macrophages, are transformed into mature HDL, and they carry cholesterol back to the liver. The recovery mechanism is enabled by the presence of the enzyme lecithin cholesterol acyltransferase (LCAT), which adds an acyl group to the 3 carbon atoms of cholesterol, making the cholesterol more fat-soluble and favoring its entry into HDL. Thanks to the enzyme called Cholesteryl ester transfer protein (CEPT), they can also exchange cholesterol with other atherogenic lipoproteins such as VLDL (Kosmas 2018).
7. Lipoprotein (a) (Lp (a)): Lp (a) is a spherical complex, with a size of 30 nm, and density between 1.055 and 1.085 g/ml. It has a similar structure to LDL, characterized by an apolipoprotein (a) attached to Apo B-100 via non-covalent interaction and a disulfide bond.

This particle is pro-atherogenic. Elevated levels of this lipoprotein are found in heterozygous familial hypercholesterolemia, and it does not derive from other lipoprotein catabolism (Krempler 1979; Maranhão 2014).

Lipoprotein	Density (g/ml)	Size (nm)	Major Lipids	Major Apoproteins
Chylomicrons	<0.930	75-1200	Triglycerides	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
Chylomicron Remnants	0.930- 1.006	30-80	Triglycerides Cholesterol	Apo B-48, Apo E
VLDL	0.930- 1.006	30-80	Triglycerides	Apo B-100, Apo E, Apo C
IDL	1.006- 1.019	25-35	Triglycerides Cholesterol	Apo B-100, Apo E, Apo C
LDL	1.019- 1.063	18- 25	Cholesterol	Apo B-100
HDL	1.063- 1.210	5-12	Cholesterol Phospholipids	Apo A-I, Apo A-II, Apo C, Apo E
Lp (a)	1.055- 1.085	~30	Cholesterol	Apo B-100, Apo (a)

Table 1. Lipoprotein Classes (Feingold 2000)

4. Lipoprotein metabolism

There are three pathways involved in lipoprotein metabolism: the exogenous pathway, the endogenous pathway and the reverse transport of cholesterol (Crook 2013). In the exogenous pathway cholesterol and fatty acids from diet, once digested, are absorbed in the intestine, where they are subjected to esterification for the production of cholesterol esters and triglycerides. Triglycerides are associated with apolipoproteins, mainly Apo A and Apo B, to form chylomicrons, which are then released into the bloodstream via the lymphatics (Olofsson 2005). Other apolipoproteins from HDL, such as Apo C and Apo E, are also incorporated into chylomicrons; in particular Apo C-II in the capillaries allows the activation of lipoprotein-lipase (LPL), thus determining the hydrolysis of triglycerides into fatty acids and glycerol. Fatty acids are used by adipocytes, myocytes, or bound to plasma albumin, while glycerol enters the hepatic glycolytic pathway.

After the removal of triacylglycerols, the particle detaches from the endothelium of the capillaries and return to the circulation where it actively exchanges apoproteins with other plasma lipoproteins. In this stage remnant chylomicrons, rich in apoB, apoE and cholesterol, are rapidly sequestered from the circulation by the liver protein, LDL-receptor-related protein (LRP), which binds Apo E. Remnants of chylomicrons are collected by the liver through receptor-mediated endocytosis. The binding between Apo E and specific receptors on the surface of hepatocytes promotes the uptake of the particle, which releases the remaining cholesterol and is then degraded in the lysosomes (Giammanco 2015).

In the endogenous pathway, an essential role is played by the liver, the predominant source of endogenous lipids. These liver products are transported into the circulation in the form of VLDL, by the protein, Microsomal Triglycerides Transfer Protein (MTTP), which allows their assembly,

conveying lipids towards apolipoprotein B-100. To become mature, the partially lipidated VLDLs are transported to the Golgi via vesicles, where they acquire Apo A-I and Apo E.

Mature VLDLs are transported from the liver to muscle and adipose tissue, in which an LPL is activated, hydrolyzing most of the TG belonging to the VLDLs in order to obtain substrates that act as a source of energy between meals, thus forming the IDL. Some of the IDLs are captured by LDL-R present on the liver, while others are subjected to the action of hepatic lipase (HL), thus forming LDL (Olofsson 2005). These latter lipoproteins can in turn be taken up by LDL-R, expressed on all mammalian cells. The bound particles, internalized by endocytosis, traffic to lysosomes, where the lysosomal acid lipase (LAL) hydrolyzes the cholesterol esters making them available for reactions, such as: the formation of membranes, the synthesis of steroid hormones, or the re-esterification by the enzyme cytoplasmic Acyl-CoA-Cholesterol-Acyl-Transferase (ACAT) to then be stored and accumulated inside the cytosolic lipid droplets (Brown 1999).

Before LDL interacts with cellular receptors, it can be modified thanks to CETP, which transfers triglycerides and esterified cholesterol between lipoproteins. The action of this protein can lead to the formation of more pro-atherogenic LDL, the small and dense LDL (Brown 1999). CETP can transport TG from VLDL to both HDL and LDL in exchange for cholesterol esters. This step reduces the amount of ester cholesterol contained within the LDL, also increasing TG, which makes the LDL more sensitive to the action of HL.

LDL-R activity is regulated at a transcriptional and post-transcriptional level, leading to three distinct and simultaneous events: 1) a decreased synthesis of cholesterol, 2) a decrease expression of LDL-R, and 3) an increased synthesis of ester cholesterol. The sterol regulatory element binding protein (SREBP) regulates these negative feedback effects by modulating the transcription of genes involved in sterol biosynthesis, such as HMGCoA-synthase, hydroxymethyl-glutaryl-coenzymeA-reductase (HMGCoA-reductase), LDL-R and other enzymes involved in the metabolism of fatty acids (Costet 2006).

The last pathway involved in the lipoprotein metabolism is reverse cholesterol transport (RCT). RCT is a physiological process by which excess cholesterol in peripheral tissues is transported by HDL to the liver for secretion into bile and feces, and will be discuss in detail in the following section.

5. Reverse Cholesterol Transport (RCT)

Almost 60 years have now passed since the introduction of the RCT concept, first proposed in the late 60's by Glomset and Ross (Glomset 1968). The reverse cholesterol transport represents a physiological process, where HDLs transfer excess cholesterol from peripheral tissues to the liver for its subsequent removal in the bile and faeces (Glomset 1968). It is a protection mechanism against

atherosclerotic diseases, promoted by HDLs, as most of the peripheral cells and tissues are unable to remove cholesterol by themselves, but exploit this ability of HDL to do so. In fact, macrophages that have engulfed cholesterol, also known as “foam cells”, are the initial phase of atherosclerotic lesion and RCT is a way to counteract the progression of atherosclerosis.

In RCT, HDL acquire free cholesterol released by cells via passive or active transport mechanisms, and transport them to steroidogenic tissues, capable of catabolizing cholesterol, or to the liver for later elimination via the faeces.

All this serves to keep cholesterol at optimal levels, because, although it is necessary for correct cellular functioning, at high levels cholesterol is toxic (Rader 2009). Therefore, RCT represents the most relevant and known anti-atherogenic function of HDL.

As previously mentioned, Glomset and Ross were the first to demonstrate the existence of a relationship between RCT and atherosclerosis (Glomset 1968); in particular, they hypothesized the role of an imbalance between the deposition and removal of cholesterol from the arteries, following an endothelial damage, for the development of atherosclerotic lesions (Ross and Glomset 1973).

The two researchers found a correspondence between the increase in HDL-C and the reduction of arterial cholesterol through the first step of RCT, namely the efflux of cellular cholesterol. HDL-C is essential in this athero-protective process (Miller 1975). Subsequent studies have shown how HDL-C levels correlate with CV risk (Collaboration E. R. F., Di Angelantonio 2009) and how lipid-lowering therapies, such as statins, may have benefits in reducing CV risk (Collaboration CTT 2017)

The amount of cholesterol removed from the cell depends on the amount of IC cholesterol, the expression of cholesterol transporters and the composition of HDL. There are four phases that characterize RCT represented in Figure 3 (Rousset 2011; Sviridov 2017).

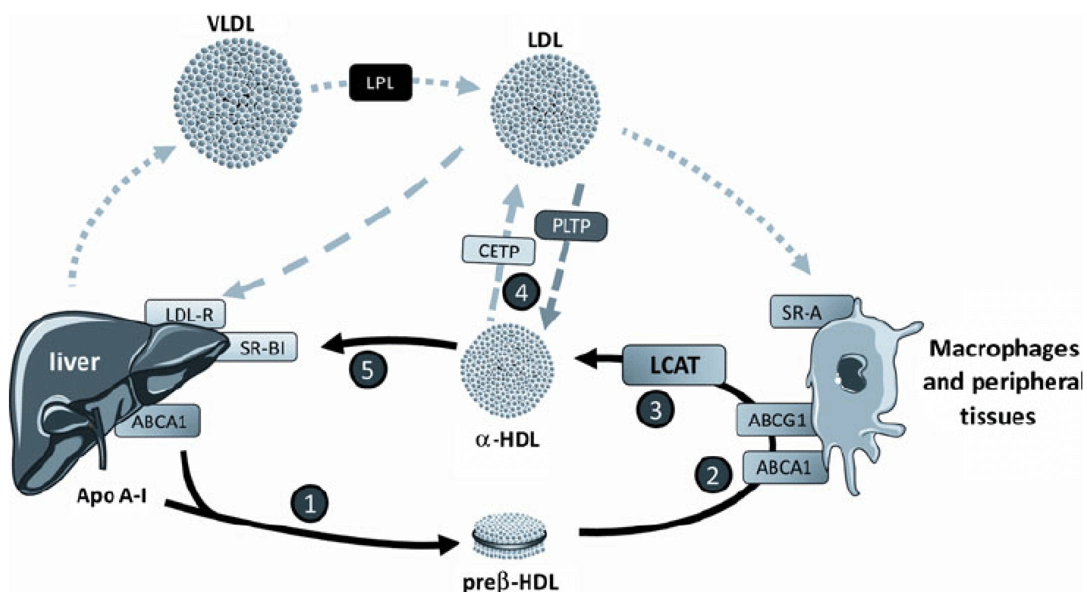


Figure 3: Schematic representation of reverse cholesterol transport (Rousset 2011)

The first phase is represented by the efflux of cholesterol in free form, released by the cells by means of transport mechanisms to HDL. In this phase, the escape of cholesterol from the cell occurs via passive transport, which does not involve energy consumption and occurs according to a concentration gradient, or via active and unidirectional transport mechanisms such as that mediated by ATP-binding cassette A1 (ABCA1) and by ATP binding cassette G1 (ABCG1). Alternatively, another transporter involved is the scavenger receptor class B type I (SR-BI) which mediates facilitated diffusion.

Subsequently LCAT intervenes to convert free cholesterol, now incorporated by HDL, defined as nascent, into ester cholesterol. This ability of LCAT is fundamental for the remodeling of HDLs, and therefore for their maturation. The action of LCAT also prevents free cholesterol from returning to the cells, favoring the maintenance of a cholesterol gradient such as to allow a continuous outflow of cholesterol.

In the third step, cholesterol ester, obtained by the enzymatic action of LCAT, is transferred via CETP from HDL to VLDL and LDL in exchange for TG. Thus, HDL are low in cholesterol ester and rich in TG.

The last phase is the uptake of cholesterol ester by the liver. This phase occurs via LDL-R present on the surface of the liver, and also cholesterol ester can return to the periphery. Alternatively, cholesterol ester present in HDL can be directly captured by the liver via SR-BI. It is a selective uptake, in which the HDL undergo, through hepatic lipase, a remodeling process, where HDL rich in TG are transformed into poor HDL, allowing the release of cholesterol ester, phospholipids, fatty acids and glycerol, which will come then absorbed by the hepatocytes.

Once it reaches the liver, cholesterol will be converted into bile salts, capable of emulsifying dietary lipids, and will be secreted by the bile. Most of the liver cholesterol is then re-absorbed in the intestine, the rest is eliminated in the faeces (Hill and McQueen 1997).

To date, methods have been developed for the quantification of RCTs in animals, while those in humans are under development. These animal testing methods provided information on molecular regulation, resulting in a stronger association with atherosclerosis than that between atherosclerosis and plasma HDL-C concentration. it is evident that the promotion of RCT can be a potential therapeutic approach for the prevention and/or regression of atherosclerotic vascular disease (Leong, Ng, and Jaarin 2015; Rader 2009).

5.1 Mechanism of cholesterol efflux capacity

Because many cell types are unable to catabolize cholesterol, the first and limiting step of RCT, the efflux of cellular cholesterol, is essential for cholesterol homeostasis. Cholesterol overload, which can occur in arterial wall macrophages, called "foam cells", is involved in atherosclerotic plaque formation (Moore 2013).

Two passive pathways are involved in the efflux of cellular cholesterol, namely simple diffusion via aqueous diffusion, and diffusion facilitated via SR-BI transporter. In addition to these two processes we find two active pathways mediated by two ABC family transporters, ABCA1 and ABCG1 (Sun 2021).

In murine peritoneal macrophages in conditions of normal cholesterol levels, about 80% of the efflux involves aqueous diffusion, while in condition of excess cholesterol, this pathway represents about 30% of the total efflux. In fact, under this condition about two thirds of cholesterol efflux occurs through active transport, where the main transporter involved is ABCA1 (Adorni 2007). Active transporters are important in situation of cholesterol loading in murine peritoneal macrophages, and a lack of these transporters leads to an accumulation of foam cells and an acceleration of the development of atherosclerosis in mice (Yvan-Charvet 2007; Sun 2021).

1)Aqueous Diffusion Cholesterol Efflux Pathway: is a simple diffusion process, that does not require energy consumption and is controlled by the concentration gradient of free cholesterol between the cell and lipoproteins, which act as cholesterol acceptors (Rothblat 1999). This passage from cell membranes can occur because cholesterol molecules are sufficiently soluble in water (Rothblat 1982). Aqueous diffusion process involves desorption of individual cholesterol molecules from the plasma membrane of, which diffuse through the aqueous phase and are subsequently incorporated into the acceptors. Once the cholesterol has passed through the membrane, it is transferred to the extracellular acceptors.

The transfer kinetics can be evaluated using rate constants for the movement of cholesterol molecules into and out of the donor and acceptor surfaces (Rosenson 2014; Yancey 2003). At low acceptor concentrations, the transfer rate depends on the diffusion-mediated collision frequency between the cholesterol molecules and the plasma acceptors. At high concentrations of extracellular acceptor, the desorption of the cholesterol molecules from the surface of the donor particles appears to be the rate-limiting step (Yancey 2003).

Since cholesterol tends to interact more with phospholipids, the factors that can reduce membrane density tend to increase the rate of cholesterol transfer. In particular, the net transfer is favored by an increase in the cholesterol/phospholipid ratio in the cell membrane and by a simultaneous reduction

of the same ratio in the acceptor (Sasahara 1998). Moreover the rate of cholesterol release mediated by the aqueous diffusion process is significantly influenced by the properties of the acceptors (Rothblat 1999). The size of the acceptors is an important factor for the diffusion process as the presence of extracellular matrix limits access to the cell surface. Large particles are inefficient acceptors and therefore small particles containing phospholipids can easily approach the cell surface, thus reducing the thickness of the aqueous layer that the desorbed cholesterol molecules must pass through (Rothblat 1999). Small particles containing phospholipids are unable to directly contact the plasma membrane due to the repulsion between phospholipids, and the presence of an aqueous layer that will oppose the movements of cholesterol mediated by this process (Linton 2019).

Therefore, aqueous desorption and diffusion are relatively inefficient mechanisms operating in all cell types.

2)ABCA1-Dependent Cholesterol Efflux Pathway: ABCA1 belongs to the family of ATP-binding cassette transporters (ABC) that uses ATP as an energy source to transport substrates, including lipids (Dean 2001).

ABCA1 is a single polypeptide chain that forms two transmembrane domains, each with six helices, followed by two ATP-binding domains (Van Eck 2005). The protein also possesses two large highly glycosylated extracellular loops that are linked in several places by disulfide bridges (Oram 2005).

ABCA1 is located at the plasma membrane and late endosomal compartments (Neufeld 2001).

ABCA1 appears to be essential in acting against the development of foam cells and atherosclerosis (Yu 2013). Incubating Apo AI with foam cells derived from macrophages, results in efflux of free FC cholesterol with subsequent formation of nascent HDL (Hara 1991). In Tangier's disease, a mutation in the ABCA1 gene causes low levels of plasma HDL, as well as poorly lipidated and rapidly catabolized Apo AI (Francis 1995; Assmann 2019; Kang 2010).

ABCA1 expression appears to be up-regulated in presence of cholesterol load, which consequently leads to an increase in the FC efflux and to the formation of nascent HDL. ABCA1 allows an unidirectional active transport of IC cholesterol and phospholipids to extracellular acceptors; In particular, this transporter actively transfers phosphatidylserine, sphingomyelin and phosphatidylcholine, with a preference for the latter (Quazi 2013). A simultaneous efflux of FC and phospholipids, deriving from both the plasma membrane and the endosomal compartments, to the lipid-free Apo A-I, is mediated by this translocase activity of the transporter (Chen 2001). Apo A-I acceptors deficient in cholesterol and depleted of PL, called preβ-HDL are lipidated by ABCA1(Duong 2006; Mulya 2007). ABCA1 has a half-life of 1-2h but binding between Apo A-I and the ABCA1 transporter inhibits calpain-mediated proteolysis, increasing the levels of ABCA1 in the plasma membrane (Yokoyama 2012).

In addition to Apo AI, the transporter is capable of interacting with apolipoproteins with amphipathic motifs, such as apoA-II, apoA-IV, apoC-II, apoC-III and apoE, which efficiently induce ABCA1-mediated transport (Van Eck 2005).
Although ABCA1 is ubiquitously expressed in all tissues, it is found at high concentrations in the liver, brain, adrenal glands and on cells important in atherosclerosis, namely macrophages. In the liver ABCA1 lipidates preβ-HDL followed by the development of nascent HDL, while in atherosclerotic plaques, ABCA1 allows the removal of lipids from macrophages deriving mainly from the ingestion of modified LDL or apoptotic cells (Tall 2008).
Preβ-HDL infusion decreases vascular inflammation thus constituting an intravenous therapy for the reduction of atherosclerosis (Van Lenten 2004, 2008). Clinical data suggesting how preβ-HDL infusions could be effective in atherosclerotic disease derive from a study on the infusion of a form of Apo A-I, known as Milano (Nissen 2003). In this study the infusion of Apo AI Milano allowed the reduction of atheroma, and this result was also confirmed by two other clinical trials (Sacks 2009; Tardif 2007).
Therefore, ABCA1 plays a decisive role at the beginning of the RCT, and the nascent HDLs, which arise from the interaction with preβ-HDL, undergo subsequent maturation mediated by LCAT. Those mature HDL will be then become a substrate for the other mechanisms of efflux (Zanotti 2012).
ABCA1 expression is regulated by multiple mechanisms, which act at various levels. As mentioned above, ABCA1 transcription is induced by cholesterol overload in cells. Furthermore, the expression of ABCA1 can be induced by the activation of the liver X receptor/retinoid X receptor system (LXR / RXR) (Costet 2000, 2003), an obligate heterodimer. Among the LXR ligands we find, for example, 22-hydroxycholesterol, an oxysterol, while among the RXR ligands there is 9-cis-retinoic acid (Costet 2000). In some cells the induction of LXR may be caused by the activation of the peroxisome proliferator-activated receptor γ (PPAR–γ). ABCA1 in murine macrophages can be induced with analogues of cyclic adenosine monophosphate (cAMP), which seems to partly involve LXR activation (Zanotti 2006).
The binding with Apo A-I regulates, at the post-transcriptional level, the expression of ABCA1, preventing its degradation, while the turnover of ABCA1 seems to be promoted by unsaturated fatty acids (Wang 2002).
Several mechanisms are proposed to explain ABCA1's ability to mediate Apo AI efflux: a first mechanism is defined as a "retro-endocytosis mechanism" in which ABCA1 binds Apo AI at the plasma membrane, internalizing it and transferring it into IC compartments, where it is lipidated and then secreted as nascent HDL (Van Der Velde 2010).

Vedhachalam and colleagues support the hypothesis that ABCA1 promotes the creation of complexes between Apo AI, phospholipids and cholesterol, with a mechanism that involves the interaction of Apo-AI both with ABCA1, allowing regulatory activities, and with membrane lipid domains that it would seem to play a major role in Apo-AI lipidation (Vedhachalam 2007).

The C-terminal region appears to play important role in mediating lipid efflux. Mutations in this C-terminal domain of Apo-AI reduce ABCA1 efflux (Favari 2002). This amphipathic domain allows binding to membrane lipid domains (Burgess 1999).

A second model proposed, called the "two-step model" says that phospholipids are first to be transferred, followed by FC. However, other researchers claim, according to the "one step model", that both lipids are transferred simultaneously (Yancey 2003).

Also exists the 3-phase model, according to which initially there is a bond between the transporter and Apo AI, which translocates phospholipids to the external side of the membrane, leading to an asymmetry of the phospholipid bilayer of the membrane with consequent distortion of the membrane. This distortion would be resolved with the formation of everted lipid domains, that bind different Apo A-I, thus solubilizing these domains leading to the formation of nascent discoidal HDLs with 2 or 4 Apo AI molecules, FC and phospholipids (Vedhachalam 2007). A more recent model is presented in Figure 4 (Phillips 2014).

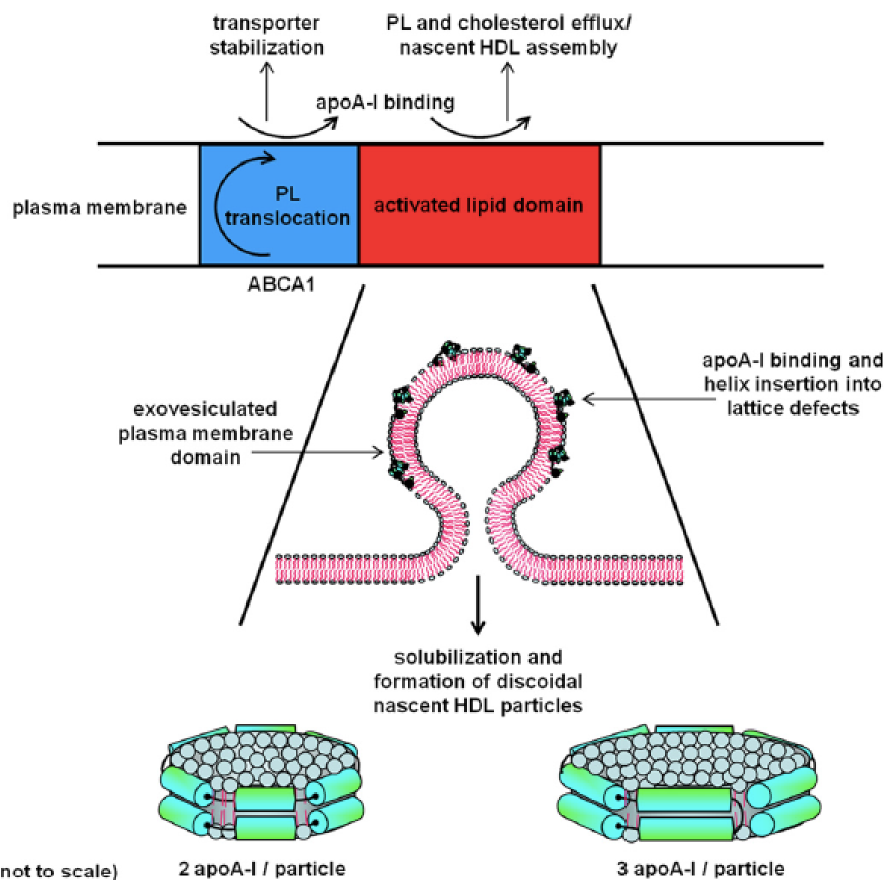


Figure 4: Summary of a proposed ABCA1 molecular mechanism (Phillips 2014)

Initially Apo A-I and ABCA1 interact, which stabilizes ABCA1 and the interaction between Apo A-I and membrane lipids, thus allowing the formation of HDL. The translocation of phospholipids by the transporter creates the activated lipid domain that binds Apo A-I. This domain is obtained starting from a flexion of the membrane, and also includes an exovesiculated segment of the plasma membrane. Starting from the fragmentation of the phospholipid bilayer, a process due to the detergent properties possessed by Apo A-I, the formation of nascent discoidal HDL particles is obtained. The HDLs, thus formed, are characterized by small double layer segments of phospholipids and cholesterol, which are usually stabilized by two or three molecules of Apo A-I (Phillips 2014).

3)ABCG1-Dependent Cholesterol Efflux Pathway: Another transporter belonging to the ABC transporter family is ABCG1. The structure (Figure 5) is characterized by an ABC domain, containing 3 highly conserved domains (Walker A, Walker B and Signature motif), as well as a transmembrane domain (TMD) containing 6 α-helices (Hardy 2017).

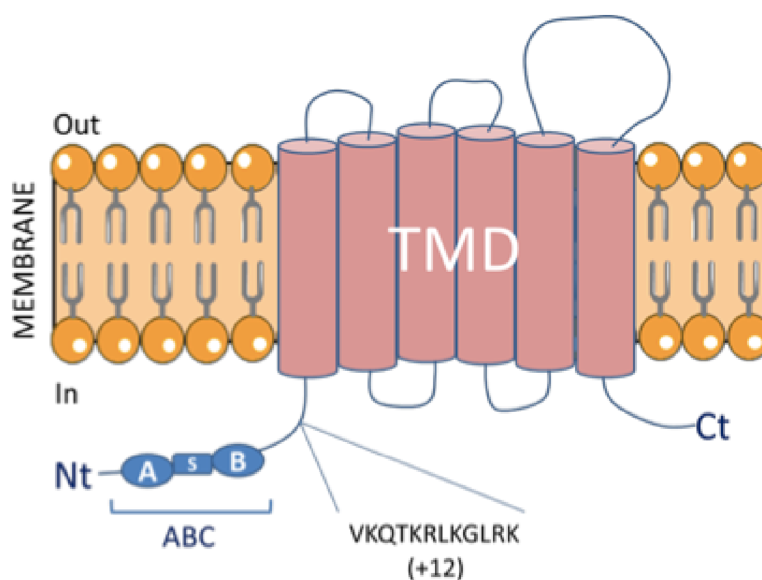


Figure 5: Structure of the membrane ATP-binding cassette G1 (ABCG1) transporter (Hardy 2017)

ABCG1 can be found in the brain, thymus, lungs, adrenal glands and spleen, while low level of this transporter is found in the liver (Oram 2006).

This transporter allows unidirectional transport of FC and phospholipids to mature HDL, like HDL2 and HDL3 (Kennedy 2005; Wang 2004), but not to free Apo A-I (Yu 2013).

Unlike ABCA1, this transport does not require a direct link with HDL (Sankaranarayanan 2009; Wang 2004), because ABCG1 seems to transport and redistribute cholesterol in the lipid membrane domains, where the uptake by mature HDLs would take place (Cavelier 2006).

As for ABCA1, also for ABCG1 the expression is increased in case of cholesterol load as well as by the nuclear receptors system of LXR/RXR (Costet 2000; Laffitte 2001). Oxysterols, obtained by

cholesterol conversion, act as ligands for LXR, stimulating the expression of both transporters, ABCA1 and ABCG1, in cells rich in cholesterol. A double role is played by LXR, because oxysterols stimulate the movement of ABCG1 at the membrane level, and as a consequence there will be an increase in the efflux of cholesterol to HDL (Wang 2006).

There is a synergy between ABCA1 and ABCG1, in particular the former would lead, starting from Apo A-I poor in lipids, to the formation of partially lipidated nascent HDL, which are then the preferential acceptors of ABCG1.

Also, ABCA1 knock out mice show a moderate increase in atherosclerosis, and this has not been found in ABCG1 knock out mice. Despite this, knock out mice for both genes show a marked increase in atherosclerotic lesions (Yvan-Charvet 2007), due to a defective outflow towards both Apo A-I and mature HDL, which also involves an increase in inflammatory responses (Yvan-Charvet 2008).

Recent interest has also been directed towards microRNAs (miRNAs), post transcriptional regulators of genes involved in physiological processes (Marquart 2010; Rayner 2010). There is a particular miRNA, known as miRNA33, which inhibits the expression in the liver of both transporters, leading to the reduction of HDL levels (Horie 2010) caused upstream by a reduced functioning of ABCA1, and therefore to a lower cholesterol efflux (Yvan-Charvet 2008). To overcome this problem, and therefore to improve the efflux of macrophage cholesterol, miRNA33 inhibitors have been formulated result in an increase in HDL-C and a reduction in atherosclerosis (Rayner 2011; Rotllan 2013).

4)SR-BI-Dependent Cholesterol Efflux Pathway: another transporter is an 82 kDa transmembrane glycoprotein, called scavenger receptor class B type I (SR-BI), located at the plasma membrane with two N and C-terminal transmembrane domains and a extracellular central domain (Williams 1999; Meyer 2013; Shen 2018).

It belongs to the family of scavenger receptors and has a 30% analogy with another receptor belonging to the same family, CD36, responsible for the absorption of long-chain fatty acids (Shen 2018).

SR-BI is expressed in liver and steroidogenic tissues, where it mediates a bidirectional transport of cholesterol and a cellular unidirectional absorption of lipids such as cholesterol ester, phospholipids and triglycerides, derived from lipoproteins (Zannis 2006). The transporter is also found expressed in the brain, intestine, kidneys, macrophages, adipocytes and endothelial cells (Van Eck 2005). The receptor can bind HDL, VLDL, LDL, AcLDL, oxidized LDL and anionic lipid vesicles (Hoekstra 2017). Furthermore, the presence of phospholipids in HDL increases the efflux through this transporter, thus allowing this transporter to facilitate aqueous diffusion, promoting the efflux of FC to acceptors rich in cholesterol (Yancey 2000; Shen 2018).

Therefore, SR-BI allows a bidirectional flow of FC between cells and HDL, the overall effect of which will be determined by various factors, such as the concentration of cholesterol in the cell, or the composition and the concentration of extracellular acceptors.
SR-BI is an important transporter not only in the first step of RCT, but also at the last phase as it is capable of depleting the lipid core of mature HDL and promoting the uptake of these lipids at the hepatocytes level (Yancey 2003; Van Eck 2005).
Various factors influence the expression of the transporter: 13 hydroxylinolenic acid (LA) and resveratrol (Res) induce the expression of this transporter (Kämmerer 2011; Voloshyna 2013), while the pregnancy-associated plasma protein A (PAPP-A), a metalloprotease involved in the insulin-like growth factor (IGF) pathway, reduces its expression causing the atherogenic formation of "foam cells" (Tontonoz 1998). Studies conducted on knock-out mice for SR-BI shows that this transporter is athero-protective, since its lack leads to an exacerbation of atherosclerosis, despite the increase in HDL-C (Acton 1996; Van Eck 2003).

6. HDL heterogeneity

High-density lipoprotein cholesterol (HDL-C) along with Apo A-I, their main protein component, are a potential predictor of acute cardiovascular (CV) events, as HDL-C levels are inversely and independently associated with acute CV risk in different patient populations (Montecucco, Favari 2015). The main protein component of HDL is Apo A-I, produced by enterocytes and hepatocytes. Another important apolipoprotein component, which accounts for 20% of the protein content in HDL, is Apo A-II, although HDL may also contain Apo A -IV, Apo AV, Apo CI, Apo C-II, Apo C-III, Apo D, Apo E, Apo J, Apo L (Feingold 2000).
HDLs are characterized by a hydrophobic core of cholesterol esters and triglycerides, surrounded by a hydrophilic outer surface layer composed of phospholipids, FC and apolipoproteins. HDLs in our bodies are remodeled by cellular and plasma factors that are responsible for the heterogeneity of this class of lipoproteins (Feingold 2000). Shape, size, surface charge, density and composition of proteins and lipids are the characteristics by which HDL differ (Rader 2002). HDLs can be classified on the base of apolipoprotein content into: Apo A-I, containing only Apo-AI, and Apo-AI / Apo-AII which contain both apolipoproteins.
Furthermore, HDLs can be classified on the base of separation by ultracentrifugation into: HDL-2 ($1.063 < d < 1.125$g / ml), large and less dense, and in HDL-3 ($1.125 < d < 1.21$ g / ml), small and dense. HDLs can also be classified on the base of size in descending order from 10.6 to 7.6 nm into five subpopulations: HDL2b, HDL2a, HDL3a, HDL3b and HDL3c (Rader 2002).

Finally, the separation with gel electrophoresis allows the subdivision of HDL into subclasses (Huang 1994; PJ 2002; Rothblat 2010), that is:

- α-HDL, larger, spherical and rich in lipids (represent the most present form);
- preβ-HDL, smaller, of discoidal form, associated with two or more molecules of Apo A-I complexed with phospholipids and free cholesterol;
- γ–HDL, small fraction of particles with migration γ containing Apo E.

These different classes of HDL are generated by a remodeling process carried out by different enzymes in the extracellular space (EC), which can lead to a change in shape, size, surface charge, lipoprotein composition (Rye 2009).

These changes can be caused by several enzymes. One is LCAT, a hydrophobic plasma enzyme, which mediates the esterification of cholesterol by allowing the transfer of an acyl group from phosphatidylcholine to cholesterol, generating the cholesterol-ester and lysophosphatidylcholine. Nascent HDLs are the preferred substrate of LCAT.

Another enzyme is CETP, a hydrophobic glycoprotein of hepatic origin, that is mainly linked to HDL; CETP allows a redistribution of lipids between the various lipoprotein fractions by taking the lipids from some plasma lipoprotein particles and depositing them into others (Rye 1999).

The nascent HDLs are discoid-shaped particles, free of cholesterol ester, which derive from the interaction of Apo A-I (free or low in lipids) with ATP Binding Cassette Subfamily A Member 1 (ABCA1), a transporter that allows the efflux of phospholipids and free cholesterol from hepatocytes and other peripheral cells. HDLs that are generated with this interaction are also called preβ–HDL and acquire cholesterol via LCAT. The action of LCAT results in mature spherical particles called α-HDL (Jonas 2000). LCAT transfers a fatty acid from phosphatidylcholine to free cholesterol, thus forming cholesterol ester, which then forms the hydrophobic nucleus of HDL. In addition, α-HDL, increases in size, due to the interaction between HDL and Apo B-containing lipoproteins. This interaction is mediated by the PLTP enzyme leading to the transfer of phospholipids between plasma and lipoproteins. PLTP also induces the fusion of more particles and promotes the loss of Apo A-I, thus leading to the formation of preβ1-HDL (Jonas 2000).

CETP can cause reconversion of mature HDLs into small via de-lipidation of large particles. CETP, circulating mainly in association with HDL, transfers cholesterol ester from lipoproteins rich in TG, containing ApoB, to HDL in exchange for TG, thus resulting in a drop in the lipid content of the HDL core.

Other enzymes such as hepatic lipase (HL), endothelial lipase (EL) also are involved in the catabolism of HDL.

HL is synthesized in hepatocytes, whereas EL has a much wider tissue distribution, being synthesized in the liver, kidneys and endothelial cells. Both cause hydrolysis of TG and in part the phospholipids accumulated by HDL. At the end of this remodeling, part of the free and ester cholesterol of HDL is taken up by the hepatocytes via specific membrane transporters such as the class B type I scavenger receptor (SR-BI).
The lipid-poor Apo A-I particles thus obtained can again act as substrate for ABCA1, or can be incorporated into discoidal or spherical HDLs, or eliminated by renal excretion (PJ 2002).

6.1 HDL functionality: a new biomarker for CV risk

HDLs have different athero-protective functions. First is the ability to promote the first step of RCT, the cellular efflux of cholesterol, but they also possess important antioxidant, anti-inflammatory, anti-thrombotic, anti- infectious, anti-apoptotic and vasodilating properties (Camont 2011).
To date, the inverse correlation between the risk of incidence of cardiovascular disease, such as atherosclerosis, and the concentration of HDL is known (Davidson 2018). From epidemiological evidence it appears that low HDL levels are a cardiovascular risk factor (Gordon 1977), thus indicating that HDLs play a protective role (Bonizzi 2021).
Although prospective observational studies have confirmed an inverse relationship between HDL-C levels and CV risk, and although some analyzes of randomized controlled trials appear to show that increased HDL-C decreases CVD risk (Toth 2013), the results of recent clinical studies aimed at increasing plasma HDL-C levels did not lead to the improvement of CVD outcomes (Voight 2012).
In addition, the lack of efficacy of Dalcetrapib, a CETP inhibitor, and niacin, aimed at increasing plasma HDL concentrations, called into question the efficacy of an increase in HDL-C in improving CVD outcomes (AIM-HIGH Investigators 2011; Schwartz 2012). Furthermore, genetic variants associated with an increase in HDL-C do not appear to show a decrease in CVD risk (Voight 2012).
These results therefore reinforced the idea that the increase in plasma HDL levels alone does not represent an effective therapeutic goal.
These observations raise concerns about HDL quality. HDLs are in fact a heterogeneous category of lipoproteins in terms of size, load and lipid content (Ossoli 2016) and show functional differences such as those concerning the ability to promote cholesterol efflux from cells, the first step of the RCT.
The cellular efflux of cholesterol can occur through different active or passive transport mechanisms and the various subclasses of HDL vary in their abilities to interact with the different cholesterol transporters. For this reason, the efficiency of HDLs in a single individual can be assessed by measuring their cholesterol efflux capacity (CEC). A study in which this variable showed an inverse

relationship with carotid mid-intimal thickening, an index of subclinical atherosclerosis, compared to plasma HDL-C levels in two distinct cohorts of subjects, suggested the role of serum CEC as an index of CV protection (Khera 2011).

Numerous studies have subsequently been published, including an observation from studies conducted by Favari et al., in which CEC inversely correlates with vascular stiffness in healthy subjects independently of HDL-C levels (Favari 2015). In a more recent meta-analysis Chengfeng Qiu and co-workers found a strong inverse dose-dependent association between HDL-CEC and incidence of CV risk in healthy individual and in patients with CVD at baseline, independently of HDL-C (Qiu 2017).

These observations suggest that HDL functionality, as assessed by CEC, may be more relevant than plasma concentrations in quantifying CV risk (Favari 2018; Bonizzi 2021). Thus evaluating CEC in reducing CV risk would improve the prediction of this risk and stimulate interest in the search for new therapies that target HDL metabolism.

7. Antibodies against Apo A-I: role in cardiovascular disease (CVD)

Thanks to its modulating effect in the inflammation, coagulation, as well as in the formation of foam cells, humoral autoimmunity plays a role in mediating atherogenesis and CVD (Libby 2014; Satta and Vuilleumier 2015). In fact, studies have highlighted the presence within the atheromatous plaque of some cell types involved in immunity, like the T-helper-1 (Th-1) cells, which would be responsive to particular antigens or autoantigens (Hansson and Hermansson 2011; Ketelhuth and Hansson 2015, 2016). This autoimmune response has been shown to be able to regulate the atherogenic process both in a pro-atherogenic and anti-atherogenic sense (Libby 2014; Tsiantoulas 2014).

Therefore, it is now known that atherosclerosis is not only a disorder caused by an altered lipid metabolism, but also by a chronic immune-mediated inflammation, localized at the level of the arterial wall, which occurs due to a translocation of immune cells, in particular monocytes, at the endothelial level. Monocytes, in fact, once differentiated into macrophages, are able to accumulate modified forms of LDL, especially oxidized ones, transforming themselves into foam cells, which constitute the initial phase of atheroma formation. These cell types are responsible for the release of pro-inflammatory factors, namely cytokines, but also chemokines and growth factors, causing a worsening of the inflammatory process, involving also the activation of endothelial cells, the proliferation of SMCs, the recruitment of other immune cells, such as Th-1, and therefore the progression of the disease (Packard and Libby 2008; Yan and Hansson 2007).

Different types of autoantibodies have been detected in patients suffering from autoimmune inflammatory diseases, such as rheumatoid arthritis (RA), anti-phospholipid syndrome (APS) and

systemic lupus erythematosus (SLE) (Haque 2008; Manzi 1997; Marai 2008; Salmon and Roman 2008). Furthermore, several autoantigens and the respective autoantibodies have been considered associated with CV risk, such as anti-phospolipid antibodies, anti-heat shock proteins (HSPs), in particular anti-HSP-60, anti-oxidized LDL antibodies and finally antibodies against lipoproteins (Roux-Lombard 2013). In particular, among the antibodies against lipoproteins associated with CVD there are the antibodies against Apo A-I (Roux-Lombard 2013), first discovered in patients with SLE (Merrill 1995). Subsequently anti Apo A-I IgG were identified at high levels also in patients with APS(Morrow and de Lemos 2007).

In 2001 six different antibodies against Apo A-I with low specificity were recognized (Abe 2001; Dinu 1998), and two years later was formulated the first hypothesis regarding the involvement of these antibodies in atherosclerotic diseases, for a probable promotion of a dysfunction at the level of HDL (Ames 2010; Batuca 2007). These antibodies would lead to the development of an environment rich in oxidative stress, as demonstrated by the inverse relationship with the activity of PON-1, a protein associated with HDL, contributing to the anti-atherogenic properties of these lipoproteins, and also with the serum total antioxidant capacity (Batuca 2009).

Vuilleumier and collaborators identified the presence of these antibodies in RA patients, associated with high levels of Ox-LDL, pro-inflammatory cytokines, in which also they were found to be predictors of CVD (Pagano 2012; Vuilleumier 2008; Yamaguchi 2007).

Although these antibodies were initially detected in patients with autoimmune diseases at risk of CVD, later on, these autoantibodies were found to be elevated in population at high CV risk, such as acute coronary syndromes, severe carotid stenosis, end-stage renal disease as well as in T2DM, were they have been overall associated with poor CV prognosis (Keller 2012; Montecucco 2011; Vuilleumier 2010). Lately, those auto-antibodies were found to be present in 20% of the general population where they were reported as an independent CV risk factor associated with worse CV prognosis and survival (Antiochos 2016, 2017).

Furthermore, anti-apoA-1 IgGs levels were found to be raised in obese subjects and associated with the presence of coronary calcification lesions (Quercioli 2012), while in metabolic syndrome patients undergoing a Mediterranean diet, anti-apoA-1 IgGs were found to predict resistance to waist circumference reduction (Carbone 2021). Corroborating these observations in humans, functional studies revealed that anti-apoA-1 IgG bear direct pro-inflammatory, pro-thrombotic, pro-arrhythmogenic and pro-atherogenic functions through TLR2, TLR4 and CD14 innate immune receptor signaling (Mannic 2015; Pagano 2012, 2016) promoting myocardial necrosis and death in vivo (Montecucco 2011; Montecucco, Braunersreuther 2015).

Therefore, the presence of high levels of these antibodies can be considered a biomarker of coronary artery lesions. In addition, the elevated levels anti-Apo A-I IgG were associated with increased plaque vulnerability (Montecucco 2011).
IgG against Apo A-I seem to be able to influence the cholesterol metabolism, as emerged from the significant inverse relationship with the levels of HDL, LDL and total cholesterol (Antiochos 2016; Bridge 2018; Montecucco 2011; Quercioli 2012).
In fact, as discovered in patients with diabetes mellitus type 2 (T2DM), these antibodies are associated with a reduction in CEC in fibroblasts (Dullaart 2019), as well as being inversely associated with passive diffusion-mediated CEC, and positively associated with ABCA-mediated CEC (Vuilleumier 2019).
It is now known that among the important factors involved in the cholesterol metabolism there are HMGCR and LDL-R (Goldstein and Brown 2015), both regulated by the SREBP-2 protein, which is encoded starting from the SREBF2 gene, that within its own sequence has microRNA 33a (miR-33a), responsible for reducing ABCA1-mediated cholesterol efflux (Marquart 2010; Najafi-Shoushtari 2010). Another effect promoted by this gene is the increase in both the absorption of LDL and the IC cholesterol synthesis, due to the increase expression of LDL-R and HMGCR.
Therefore, if on the one hand there is an increase in the synthesis of cholesterol, on the other there is a decrease in the release of cholesterol from the cells, resulting in a mechanism that facilitates the development of atheromatous plaque.
Factors capable of modulating LDL-R, HMGCR or miR-33a can influence lipid metabolism, acting on lipid absorption, cellular cholesterol synthesis and cellular cholesterol efflux, and would also be able to influence the formation of atheromatous plaque. For this reason it would seem that anti-ApoA-1 IgG can reorganize lipids at the cellular level, thus affecting CV risk, thanks to their influence on regulators of cholesterol metabolism.
Of interest is a recent study conducted by Pagano and collaborators on pediatric patients suffering from familial hypercholesterolemia (FH), in which the presence of anti-ApoA-1 IgG is associated with a positive lipid picture, characterized by lower levels of total cholesterol, LDL-C and miR-33a (Pagano 2019), despite the high CVD risk of this pathology, as previously demonstrated (Antiochos 2016; Bridge 2018; Montecucco 2011; Quercioli 2012). In this study, it was discovered that these autoantibodies are able to stimulate the expression of SREBP-2, LDL-R and HMGCR thus causing an increase in the synthesis of cholesterol and the entry of LDL into the macrophage cells. Furthermore, on one hand anti-ApoA-1 IgG would appear to be able to decrease the efflux mediated by passive diffusion and the expression of miR-33a, while on the other hand they would lead to an increase in the expression of the ABCA1 protein, through a feedback mechanism, which is still

insufficient to compensate the pro-atherogenic mechanisms induced by these autoantibodies (Pagano 2019).
Pagano and co-workers are studying the effect of anti-apoA-1 IgG in patients suffering a metabolic-based disorder, namely non-alcoholic fatty liver disease (NAFLD), hypothesizing their involvement in the progression of the steatosis. In fact, by modifying the mechanisms involved in lipid regulation, these antibodies play a metabolic role. Found at relevant levels also in NAFLD patients, anti-apoA-1 IgG seem to be involved in the induction of an inflammatory response, as well as in the accumulation of cholesterol through the activation of SREBP-1 in human liver cells.

8. Lipoprotein (a)

Lipid disorders can be caused not only by altered levels of LDL-C, HDL-C, TG, but also of another lipoprotein called, Lipoprotein (a) (Lp (a)). Indeed, increased Lp (a) levels is considered as a risk factor for several form of CVD (Collaboration E. R. F., Erqou 2009; Kamstrup 2016; Nordestgaard 2010).

8.1 Lp (a) structure and Apo (a) isoform

Considered an LDL-like particle, Lp (a) is a plasma lipoprotein (Mach 2020), characterized by a lipid nucleus containing Apo B-100 linked via disulfide bond to Apolipoprotein (a) (Apo (a)) (Nordestgaard 2010).
The central lipid nucleus contains cholesterol and TG esters surrounded by phospholipids, FC and a single molecule of Apo B (Nordestgaard 2010).

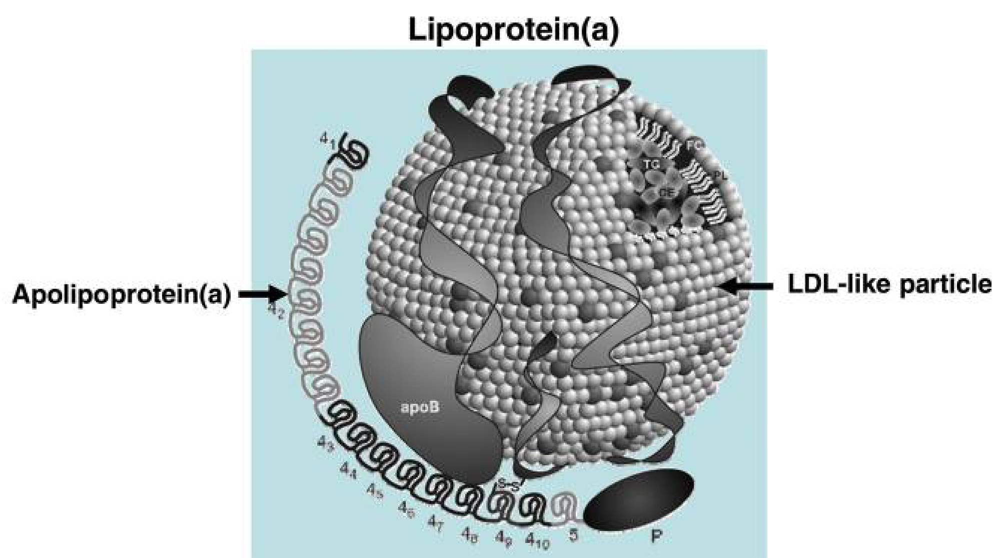


Figure 6: Lipoprotein (a) structure (Nordestgaard 2010)

In particular, the structure (represented in Figure 6) is characterized by a disulfide bond between Cys4326 of Apo B and Cys1568 of Apo (a) (Brunner 1993; Callow and Rubin 1995; Koschinsky 1993).

The Apo (a) component has a high homology with plasminogen, from which it is thought to have evolved over time as a result of mutations (Schmidt 2016). The plasminogen structure features Kringles domains (KI to KV), highly glycosylated three-dimensional heavy chain, as well as a protease domain (McLean 1987).

Instead, Apo (a) has 10 different subtypes of KIV, of which KIV_1 and KIV_{3-10} are present in a single copy, while KIV_2 is present from 2 to 40 or more copies (Van Der Hoek 1993; Lackner 1991; Maranhão 2014). In addition to KIV, Apo (a) is formed by single copy KV and an inactive protease domain (Maranhão 2014).

KIV_2 determins the isoforms of Apo (a), which differ from each other in length.

An inverse relationship was observed between the size of Apo (a) and the concentration of Lp (a) (Rader 1994), showing how the size of KIV_2 determines the concentration of this lipoprotein. In particular, repetitions lower than 22 lead to a greater plasma concentration of Lp (a) (Kostner 2017), as the smaller particles would likely be released into a circulation more easily by the hepatocytes than the larger ones (White 1997).

Lp (a) levels are also determined by single nucleotide polymorphisms (SNPs) affecting the *lpa* gene (Clarke 2009). These polymorphisms include the SNP rs10455872 and rs3798220 (Clarke 2009), splice variants G4925A and G4733A (Coassin 2017; Schachtl-Riess 2021). Furthermore, a nonsense variant, called R21X, can reduce the levels of Lp (a) by 11.7 mg / dL (Di Maio 2020).

About 30% of Lp (a) levels are determined by SNPs in the *lpa* gene, the remaining part
is caused by the different Apo (a) isoforms (Clarke 2009).

Therefore, it would seem that Apo (a) size is related to cardiovascular risk. A meta-analysis has shown that a higher risk of coronary heart disease (CHD) is present in subjects with smaller Apo (a) isoforms (Collaboration E. R. F., Erqou 2009).

Alternatively, Gudbjartsson and collaborators attribute the effect of Lp (a) to the molar concentration (Gudbjartsson 2019). Another 2019 study also showed that, although the isoform is inversely associated with Lp (a) levels, there is no link with CV risk (Paré 2019).

Among the variants in the LPA gene, the intron G4733A within KIV2 is responsible for a permanent reduction in Lp (a) levels by 13.6 mg / dL, with a 9% CAD risk reduction (HR = 0, 91; 95% CI 0.89-0.93) (Koschinsky and Boffa 2021).

This demonstrates that Lp (a) levels are better predictors of CV risk than the size of Apo (a) isoforms or variants of the LPA locus.

8.2 Lp (a) synthesis, metabolism and catabolism

Lp (a) does not result from the catabolism of other lipoproteins (Krempler 1979). In vitro studies show that the main site of Apo (a) synthesis is in the liver. The site of lipoprotein assembly is not yet confirmed, but hypotheses are that it occurs intracellularly (Bonen 1997; Frischmann 2012) or extracellularly, in the circulation level or at the hepatocyte surface (Demant 2001; Lobentanz 1998). The assembly would involve the attachment of the lipid nucleus to Apo B-100 via disulfide bridge with KIV_9 of Apo (a) (Koschinsky 1993). Furthermore, Lp (a) takes longer than LDL to be cleared, in particular 11 days for Lp (a) versus 4 days for LDL, probably because the larger size of Lp (a) reduced affinity for LDL-R, making further elimination pathways necessary (Diffenderfer 2016).
Given the role of Lp (a) as a cardiovascular risk factor, it is important to understand the mechanisms that can facilitate its elimination. The main site of Lp(a) catabolism is the liver (Cain 2005), but the kidneys are also partially involved (Kronenberg 1994; Reblin 2001). There are several receptors involved in the elimination process of this lipoprotein (McCormick and Schneider 2019); LDL-R may be one, given the similarity with LDL, however, studies show conflicting results. Statins, which increase LDL-R expression, do not reduce Lp (a) levels (Boonmark 1997), while proprotein convertase subtilisin/kexintype 9 inhibitors (PCSK9i), such as alirocumab, which increase levels of this receptor, reduce this lipoprotein (Kotani and Banach 2017; Watts 2020). However, in vivo animal and human studies show that LDL-R deficiency in patients with familial hypercholesterolemia (FH) causes an increase in Lp (a) levels (Chemello 2020). Further studies are therefore needed to define the role of this receptor in Lp (a) clearance. Other receptors are involved in the uptake of Lp (a), such as asialoglycoprotein receptors, megaline receptors and macrophage scavenger receptors (Argraves 1997). Obviously, the excessive uptake of these lipoproteins by macrophages would favor the development in foam cells, which, as already mentioned, is a main mechanism of atherogenesis.

8.3 Lp (a): Cellular Effects and Molecular Mechanisms

The physiological role of Lp(a) still remains unknown. One hypothesis suggests the involvement of Lp (a) in mediating wound healing, as this lipoprotein has been detected in wounds during the healing phase (Yano 1997), and a study revealed the presence of proteins associated with Lp (a) in this process (von Zychlinski 2011). On the other hand, its involvement in multiple atherogenic processes is well known, and high levels of Lp (a) are associated with CVD risk (Clarke 2009; Collaboration E. R. F., Erqou 2009; Forbes 2016; Willeit 2018).

A feature in common with LDL, which also confers pro-atherogenic characteristics is the propensity to oxidaze and accumulate in the arterial wall, both factors that facilitate the formation of foam cells (Argraves 1997; Umahara 2011). Lp (a) might be more atherogenic than LDL due to the Apo (a) component, which is capable of inducing the atherothrombotic process with an inflammatory stimulus due to the presence of oxidized phospholipids (OxPL), accumulating in the arterial wall due to sites of lysine binding, and inhibiting fibrinolysis mediated by the inhibition of plasminogen activation (Spence and Koschinsky 2012).

OxPLs, present at the lysine binding site of the Apo (a) KIV_{10} domain, would plays an important role in the lipid portion of Lp (a) (Bergmark 2008; Leibundgut 2013; van der Valk 2016); OxPLs are pro-inflammatory as they are capable of inducing the release of proinflammatory cytokines in the artery. This is shown by studies using antibodies against OxLDL or recombinant Apo(a) constructs to mitigate the effect (van der Valk 2016). In addition, OxPLs are responsible for other pro-atherogenic effects of Lp (a), such as increased expression of inflammatory genes, release of interleukin-8 (IL-8) (Scipione 2015), and release monocyte chemoattractant protein-1 (MCP-1) which facilitate entry of Lp (a) into the vessel walls (Wiesner 2013). Furthermore, OxPL, through the uptake by scavanger receptors, would facilitate the formation of foam cells (Argraves 1997).

OxPLs induces endothelial damage (essential in the process of atheroma formation), by increasing cell membrane permeability. This has been demonstrated in human umbilical vein endothelial cell (HUVEC) monolayers treated with Apo (a) containing the lysine biding site. This effect is not noticed with Apo (a) lacking this binding site (Cho 2008). In addition, in vivo studies on animal models show that the absence of this binding site reduces the aortic lesion compared to wild type Apo (a) (Boonmark 1997).

Lp (a) can promote thrombotic processes by acting in several steps:

- Platelet activation/aggregation. It has not been defined yet how it affects these processes. The results regarding the involvement of this lipoprotein in the initial platelet activation are scarce, even if Lp (a) and apo (a) seem to be able to promote this phase through the thrombin receptor activated hexapeptide (TRAP) (Rand 1998). Furthermore, although Lp (a) and apo (a) have no impact on the aggregation promoted by collagen and thrombin, studies show that they are able to stimulate this response in presence of arachidonic acid, as well as TRAP (Martínez 2001; Rand 1998). Lp (a) also inhibits platelet aggregation with low concentrations of collagen (Gries 1996), as well as in presence of platelet activation factor (PAF) (Tsironis 2004). A double inhibition of LP (a) was also detected in the PAF system, since, although this lipoprotein is associated with PAF-acetylhydrolase (PAF-AH) (Karabina 1996), the inhibitory effect remains even after the removal of PAF- AH (Tsironis 2004).

• Pathway of the tissue factor (TF). Treatment of monocytes with Lp (a) doubles the production of TF (Sotiriou 2006), a tissue factor favoring blood clotting (Mackman 2004). This effect is caused by αMβ2 integrin activation and by the nuclear factor kappa β (NFκβ) signaling pathway (Sotiriou 2006). This prothrombotic process can also be stimulated by binding and inhibiting the tissue factor pathway inhibitor (TFPI).

• Alteration of plasminogen activation. Precisely, due to its homology to plasminogen, Lp (a) seems to inhibit the formation of plasmin, an active form obtained from plasminogen through the formation of a ternary complex, in which the plasminogen binds the tissue plasminogen activator (tPA) and fibrin. Lp (a) competes for the binding with fibrin, plasminogen and tPA, thus causing thrombosis, which can prevent the physiological degradation of clots (Loscalzo 1990; Rouy 1991). Lp (a) can inhibit plasminogen activation associating with specific inhibitors of the ternary complex components.

• Inhibition of the activation of transforming growth factor beta (TGFβ). Lp (a) can prevent the activation of TGFβ, a plasmin substrate (Lyons 1990), consequently increasing both the proliferation and the migration of cells in the vessel wall (Kojima 1991). This growth factor can protect on one hand from atherosclerosis, inhibiting the migration of SMCs (Azuma 1996), while on the other hand it would stimulate development of atherosclerosis through inhibition of EC migration and induction of intercellular adhesion molecule-1 (ICAM-1) expression on EC (Suzuki 1994).

Lp (a) is involved in vascular remodeling at the level of atheromas, where this lipoprotein accumulates (Sotiriou 2006), through changes in the proliferative and migratory capacity of resident EC/SMC cells, steps that characterize the development of atherosclerosis (Ross 1999).

Lp (a) is also responsible for the development of calcific aortic valve stenosis (CAVS). An LPA SNP rs10455872 is associated with the risk of CAVS, as well as with high levels of Lp (a) in different racial groups (Arsenault 2014; Thanassoulis 2013; Vongpromek 2015). Furthermore, in the ATRONOMER trial it was shown how the levels of this lipoprotein are associated with the progression of CAVS, as well as being associated with OxPL and a faster progression of CAVS (Duell 2016).

8.4 Lp (a) levels, methods to quantify and how to treat

Studies show that Lp (a) levels above 30 mg/dL are associated with an increased risk of myocardial infarction (Kostner 1981), data subsequently confirmed by meta-analysis, which highlighted an increase in this risk from 24 mg/dL (Collaboration E.R.F., Erqou 2009).

Kostner and co-workers show that values above 50 mg/dL lead to more than double the increase in CV risk (Kostner 1981), while Riches and colleagues associate a doubling of CV risk with Lp (a) values above 20 mg/dL (Riches and Porter 2012).

Therefore, it can be said that the higher the levels of this lipoprotein are, the greater the risk of CVD is. The European Atherosclerosis Society (EAS) and the Canadian Cardiovascular Society consider optimal Lp (a) levels below 50 mg/dL (Arnett 2019, Pearson 2021). It should be noted that about 70% of the population has Lp (a) levels below 30 mg/dL.

Generally, tests involving the quantification of Lp (a) are based on the quantification of Apo (a) via monoclonal antibodies directed against this fraction. Techniques used for the quantification of Lp (a) are: Enzyme-Linked Immunosorbent Assay (ELISA), non-competitive ELISA, latex immunoassays, immunonephelometric, immunoturbidometric and fluorescence assays (Marcovina 2003). Techniques that involve the use of electrophoresis gel can also be used, even if the accuracy of these techniques is not high.

Evaluation of the amount of Lp (a) in patients with specific clinical conditions, such as: premature CVD or family history of premature CVD, high Lp (a) levels, FH, subjects undergoing lipid-lowering treatments with recurrent CVD and those with ≥3% 10-year risk of fatal CVD is recommended according to the European guidelines (Graham 2007).

Several pharmacological treatments have proved to be useful in reducing Lp (a) levels. PCSK9i, in addition to having effects on LDL-C, are able to reduce Lp (a) levels by 20-30%, as demonstrated with both alirocumab (Bittner 2020; Gaudet 2014; Mahmood 2020; Schwartz 2020) and evolocumab (Desai 2013; O'Donoghue 2019; Raal 2014, 2016). In patients from the ODYSSEY OUTCOMES trial, treatment with alirocumab was resulted in Lp (a) reduction by 5 mg/dL with a 3% reduction in CV risk (Bittner 2020).

Another PCSK9i, called evolocumab, can also increase the expression of LDL-R, to reduce not only LDL-C, but also Lp (a) (Desai 2013; Raal 2014).

On the contrary, recent meta-analysis statin studies show that this treatment would increase Lp (a) levels by 9 to 20% (Tsimikas 2020). This effect could be due to the increase in the expression of LPA mRNA and to the increase in the secretion of Apo (a) by human hepatocytes, as obtained from in

vitro studies (Tsimikas 2020). In addition, atorvastatin seems to increase the levels of OxPL present in particles containing Apo B (Fraley 2009).

Emerging therapies for the reduction of Lp (a) levels include antisense oligonucleotides (ASOs) capable of binding to target mRNAs, such as those for Apo (a) or Apo B. ASO for Apo (a) inhibiting the synthesis of this component, prevents the assembly of Lp (a) (Tsimikas 2017), additionally ASO against Apo B, such as mipomersen, reduces Lp (a) (Merki 2008). In particular, mipomersen seems to be able to reduce Lp (a) levels by 21-30% in FH patients (Raal 2010; Santos 2015).

Moreover, niacin, with its positive effects at lipid levels, can reduce Lp (a) levels (Carlson 1989) through a dose dependent mechanism, with a reduction of 20%-40% with dosages ranging from 1.5 to 3 gr (Capuzzi 1998; Goldberg 2000). The AIM-HIGH study obtained 21% reductions in Lp (a) levels (Boden 2011), although no decrease in CV risk was found.

Finally, a non-pharmacological therapy called LDL-apheresis, can reduce Lp (a) levels by more than 50% in patients with FH (Norata 2013).

9. Non-alcoholic fatty liver disease (NAFLD)

9.1 General aspects, epidemiology and risk factors

Non-alcoholic fatty liver disease (NAFLD) is one of the most common form of chronic liver disease worldwide (McPherson 2015; Singh 2015). It is a pathology characterized by TG accumulation greater than 5% in hepatocytes, in the absence of secondary causes of liver disease (Eslam 2020).

It is a disease that progressively develops from a condition characterized only by the accumulation of fats in the hepatocytes without signs of hepatocellular damage, known as steatosis, to a state characterized not only by hepatic steatosis, but also by the presence of inflammation, necrosis and fibrosis, named non-alcoholic steatohepatitis (NASH). This can further evolve into liver cirrhosis, where the liver progressively loses its efficiency due to the replacement of functional tissue into scar-like tissue. The last, most advanced, stage of the disease is the hepatocellular carcinoma (Neuschwander-Tetri 2003; Tiniakos 2010).

NAFLD appears to be the main cause of variation in liver function indices in adulthood, childhood and adolescence. The global prevalence of this pathology is 25% (Williams 2011; Younossi 2016), with highest peaks in the Middle East, South America, Asia, Europe, North America, and partly also in Africa (Vernon 2011).

The most worrying data come from the pediatric range, where the metabolic syndrome and obesity are increasingly growing, not only in countries like the United States, but in recent years also in Italy (Dunn 2008; Welsh 2013).

Given the high presence of this disease, an early diagnosis is important to prevent progression and the onset of complications.
NAFLD is a multifactorial pathology, associated with genetic and environmental factors. Surprisingly, despite being a disease that mainly affects the liver, only a minority of subjects with NAFLD die of liver disease. CVD is the main causes of death, followed by malignant diseases, which, once again, do not affect the liver, and finally hepatic complications (Angulo 2015; Ekstedt 2015).
NAFLD is caused by the traditional risk factors for CVD, which can be grouped together by the metabolic syndrome. NAFLD is considered to be the expression of metabolic syndrome in the liver. Therefore, visceral obesity, glucose intolerance, T2DM, dyslipidemia, and hypertension are some of the common risk factors not only in CVD and in metabolic syndrome, but also in NAFLD (Angulo 2002; Ratziu 2010). Not only do the pathologies associated with metabolic syndrome constitute a risk factor for NAFLD, but NAFLD becomes a risk factor for the onset of the metabolic syndrome. This underlies a direct relationship between the accumulation of lipids within hepatocytes and the progressive increase in the severity of the typical components of the metabolic syndrome (Kotronen 2007).
Therefore, the risk factors of NAFLD are also those of CVD. In addition to the classic risk factors mentioned above, we find others considered non-traditional, such as: hyperuricemia (Li 2009), hypoadiponectinemia (Hui 2004), hypovitaminosis D (Targher *NMCD* 2007), chronic kidney diseases (CKD), pro-inflammatory markers (IL-6, PCR, TNF-α, other acute phase liver proteins), pro-coagulating factors (fibrinogen, Plasminogen activator inhibitor-1) and adhesion molecules (such as vascular adhesion protein 1) (Targher 2009; Vanni 2015).

9.2 Clinical evidence and diagnosis

The course of NAFLD is often asymptomatic, but symptoms can be as varied as malaise, fatigue and discomfort in the right hypochondrium. In some cases, hepatomegaly can occur, while in more advanced stages, splenomegaly and cirrhosis clinical signs can occur (Alba and Lindor 2003).
Common laboratory tests may detect increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), or alkaline phosphatase and γ-glutamyltransferase (GGT) (Angulo 2002). Among these the best parameter associated with NAFLD would be ALT. It is more related to hepatic fat content (Kotronen and Yki-Järvinen 2008), and several studies show an association between elevated ALT levels and CV-related mortality (Schindhelm 2007; Yun 2009).
Despite this, Bjornsson and colleagues have shown that patients with advanced NAFLD can possess normal ALT levels (Björnsson and Angulo 2007). Therefore, ALT levels cannot be the only diagnostic test performed (Vernon 2011).

Transferrin saturation and serum ferritin levels, which are increased by 15% in NAFLD patients, may also be evaluated for NAFLD diagnosis (Vernon 2011).
Elevated serum levels of anti-nucleus (ANA) and anti-smooth muscle (SMA) antibodies, which can be increased in about 30% of NAFLD patients can be diagnostic (Björnsson and Angulo 2007).
The existence of secondary causes of steatosis through biohumoral and serological assessments must be rolled out (Dyson 2014). Diagnosis requires a liver ultrasound, which can identify the presence of that "bright" region of the liver that is typical of hepatic steatosis. It is a first-line diagnostic technique whereas NAFLD is suspected, because is cheap, non-invasive and well tolerated (Ballestri 2012; Lomonaco, 2011).
This technique is only able to detect forms of steatosis affecting near 30% of liver (Dyson 2014). Therefore, techniques with greater sensitivity and reproducibility in quantifying lipid accumulation, such as computed tomography (CT) and especially nuclear magnetic resonance (NMR) are much better. However, these techniques are not typically used in the clinic due high levels of radiation (Loria 2010).

9.3 Mechanisms involved in NAFLD pathogenesis

To describe this multifactorial pathology, several hypotheses have been formulated over the years. The most popular is the "two hits" hypothesis, formulated in 1998 by two scientists, Day and James (Day and James 1998). According to this theory, the accumulation of lipids in the liver from different origins together with insulin resistance would cause the initial anomalies of this disease. This would sensitize the liver, predisposing it to the next step, consisting of the inflammatory insult and fibrogenesis, typical of NASH, and all the consequences thereby (Peverill 2014). However, NALFD is a complex disease that cannot be explained so simply. This has resulted in the generation of a new "multi-hit” hypothesis, where additional factors would be involved in the development of NAFLD. This might involve such as genetic factors, eating habits and the environment, which would induce insulin resistance, obesity, and changes in the intestinal microbiota (Buzzetti 2016).

First hit

The evolution of the NAFLD disease is characterized by an excess of TG in the cytoplasm of hepatocytes, present in form of fat droplets.
These droplets must be present in at least 10% of the hepatocytes (Neuschwander-Tetri 2003). This accumulation of TG in the hepatocytes is due to several mechanisms, including: an imbalance in the release of free fatty acids (FFA) and TG in the liver, a reduction in the use of FFA, a decrease in the export of TG from the liver and an impaired beta oxidation of FFA (Browning and Horton 2004;

Lewis 2002). On the contrary, a lower accumulation of fat in the liver occurs in the case of direct absorption of fatty acids from the diet (Donnelly 2005). Furthermore, excess carbohydrates are inducers of de novo fatty acids synthesis in the liver.
Insulin resistance, partly responsible for the accumulation of fat in the liver, is also a important in the onset and evolution of NAFLD. Insulin resistance is a reduced sensitivity of tissues to endogenous insulin with consequent compensatory hyperinsulinemia (Lonardo 2005).
There are two main forms of insulin resistance: 1) one of hepatic origin, characterized by a continuous leakage of glucose from the liver, an increase in VLDL loaded with TG, and consequently high levels of TG in the circulation and reduced levels of HDL-C; 2) another form is a peripheral one, associated with an increase in lipolysis in visceral adipose tissue (VAT), characterized by high fasting plasma levels of non-esterified fatty acids (NEFA). Thus, both the increased glucose and NEFA, as well as the high insulin levels, contribute to the synthesis and accumulation of TG in the hepatocytes (Krawczyk 2010; Vanni 2010).
As previously stated, there are different mechanisms leading to the accumulation of TG in the liver (Figure 7). As a direct consequence of insulin resistance in the adipocytes, there can be an increase in the influx of fatty acids from adipose tissue, and an increase in the lipolysis from VAT. This greater absorption by the liver of VAT-derived fatty acids, is due to the fact that this tissue carries fat to the liver and, moreover, because the adipocytes of this site, are subject to greater lipolysis (Larter 2010). Accumulation of TG in the liver is also caused by the reduction in the release of fatty acids outside this organ. The presence of elevated TG levels in NAFLD patients is linked to elevated VLDL circulating levels. Apo B-100 transfers TG into VLDL, and is deficient in NAFLD patients due to increased insulin-promoted degradation, probably by an autophagy-dependent mechanism (Ginsberg 2009). Therefore, due to the lack of Apo B-100, the release of TG from the liver is insufficient to rebalance liver levels (Koo 2013).
Accumulation of hepatic TG can also be caused by the increase in de novo synthesis of hepatic lipids. In this case insulin, in particular hyperinsulinemia, plays a decisive role, being able to activate hepatic lipogenesis by activating the Sterol Regulatory Element-Binding Protein 1c (SREBP-1c) transcription factor, which induces the transcription of other lipogenic genes, such as Fatty Acid Synthase (FAS) and Acetyl Coenzyme A Carboxylase (ACC1) (Koo 2013).
In mice SREBP-1c is induced by insulin after meals and by high-fat diets in a liver X receptor alpha (LXR alpha) dependent process (Chen 2004).
SREBP-1c is also activated by the mammalian target of rapamycin (mTOR), and inhibited by protein kinase A (PKA), AMP-activated protein kinase (AMPK), and salt inducible kinases (SIK) (Lu 2006; Porstmann 2008; Yoon 2009). Additionally, a state of hyperglycemia activates another transcription

factor, carbohydrate Response Binding Protein (chREBP), a regulator of glycolysis in the liver. chREBP activates liver-type pyruvate kinase (L-PK), which leads to formation of Krebs cycle substrate, pyruvate, from phosphoenolpyruvate. chREBP can regulate other lipogenic genes (Uyeda and Repa 2006).

In vivo studies on obese and insulin resistant mice demonstrated the notable presence of SREBP-1c and ChREBP in the liver. Furthermore, through the reduction of these two transcription factors, it was possible to demonstrate a decrease of hepatic steatosis in mice. These latest data highlight the importance of these transcription factors in two hepatic phenomena, the accumulation of TG and de novo synthesis of lipids (Dentin 2006; Shimomura 1999).

Another mechanism that facilitates the increase in TG levels in the liver is the reduction of beta-oxidation of fatty acids in mitochondria. This process converts fatty acids into acetyl-CoA. Acetyl-CoA can be converted into ketone bodies or can be inserted into the TCA cycle where it can undergo a complete oxidation (Eaton 1996).

After being brought to mitochondrial membrane, by carnitine palmitoyl transferase (CPT), fatty acyl-CoAs move to the external side of the mitochondrial membrane, where they become fatty acyl-carnitines by the action of CTP1. Th fatty acyl-carnitines are then transported to the intermembrane. At this point CPT2 converts fatty acyl-carnitines into fatty acyl-CoAs, making them available for beta oxidation in the mitochondrial matrix. Specific acyl-CoA dehydrogenases, such as MCAD, VLCAD and LCAD, beta dehydrogenate the acyl-CoA ester. Therefore, these enzymes together with beta oxidation of fatty acids are involved in the determination of the TG content in the liver. This has been demonstrated by studies on mice lacking these dehydrogenases, in which the development of hepatic steatosis was evident (Tolwani 2005).

PPAR-α together with its coactivator, PPAR gamma coactivator 1 alpha (PGC-1α) are considered important as they can increase the expression of genes such as CPT1, LCAD, MCAD (Reddy and Hashimoto 2001). These genes can also be modulated by activation of AMPK and sirtuins (Foster 2012; Houtkooper 2012).

SREBP-2 is upregulated in the development of NAFLD, due to both hyperinsulinemia and pro-inflammatory cytokines, such as IL-1 and TNF-α. The increase in LDL entry into the liver and the consequent increase in cholesterol synthesis is caused by SREBP-2, which at the nuclear level allows the expression of LDL-R, as well as the HMG-CoA reductase enzyme.

SREBP-2 also can suppress the beta oxidation of fatty acids and the cellular cholesterol efflux, thus leading to an increase in hepatic cholesterol levels, with a consequent hepatocyte death, inflammation and further overexpression of SREBP-2 (Van Rooyen 2011).

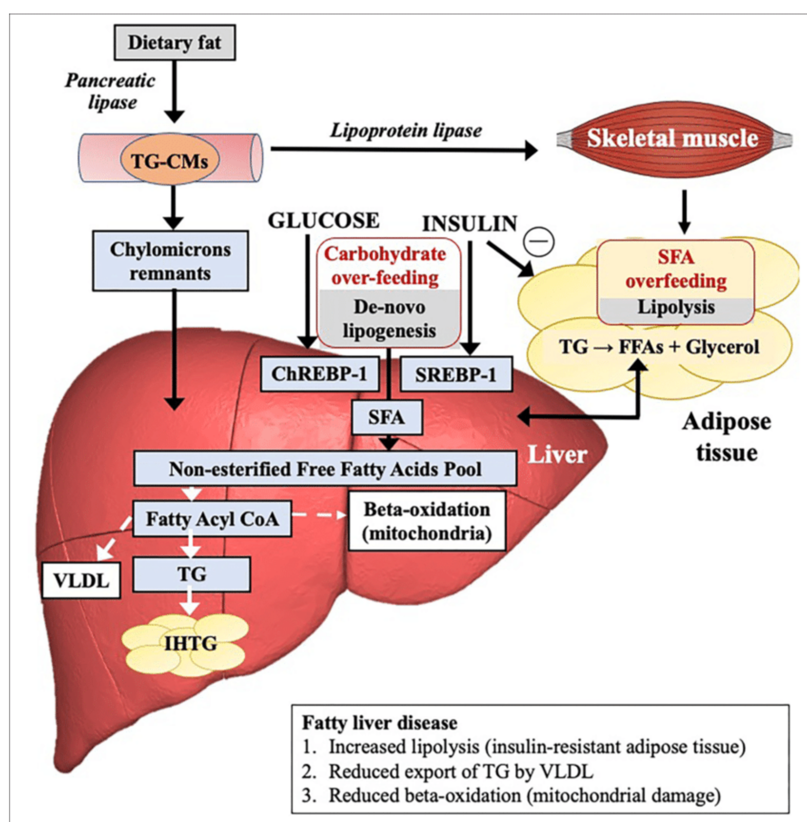


Figure 7: The mechanistic pathways leading to the accumulation of intrahepatic triglycerides and influence of fat or carbohydrate overfeeding on these pathways (Hydes 2021)

Second hit

A liver characterized by TG accumulation is more prone to the progression from NAFLD to NASH than a healthy liver. As the disease progresses, the liver is more sensitive to various stress factors such as reactive oxygen species (ROS), adipokines and cytokines. In addition to these elements, another typical characteristic of fatty liver is the impairment of its regenerative capacity, demonstrated by studies on obese mouse models, which, in the presence of fatty liver, have a lower ability to eliminate toxins than non-obese mice (Yang 1997).

Although there is no certainty regarding the factors determining the transition from NAFLD to NASH, there is a strong association with the duration and severity of the TG accumulation in the liver and of hyperinsulinemia (Basaranoglu and Neuschwander-tetri 2006).

Two other factors that could induce this progression are oxidative stress and lipotoxicity, both of which are responsible for the release of pro-inflammatory cytokines. Oxidative stress consists of an imbalance between oxidizing substances, such as ROS, and a decrease in antioxidant ones, leading to the induction of oxidative damage. Specially, the increase in ROS is caused by the increase in beta-oxidation at the level of the mitochondria and by the beta oxidation of fatty acids in peroxisomes. ROS have an effect at the cellular level due to the lipid peroxidation they cause there, but they can

also have extracellular effects, being able to form aldehydes that can diffuse from the site of origin (Browning and Horton 2004). The result is the alteration of the mitochondria both at a structural level with the formation of megamitochondria and paracrystalline inclusions, and at a functional level, because there is a reduced oxidative capacity of fatty acids and there is also an insufficient production of ATP due to a reduction in the activity of respiratory chain. Additionally, there is a lipid accumulation and oxidative stress (Vanni 2010).

Through the activation of NF-kB, oxidative stress results in an increase in the production of inflammatory cytokines, such as TNFα, IL-6, IL-8, TGFα (Byrne 2010).

Another typical element of the second hit is lipotoxicity. The adipose tissue is known to be a source of metabolic and inflammatory mediators, and cytokines produced and released by adipose tissue, known as adipokines, can have both anti-inflammatory effects, as in the case of adiponectin, and pro-inflammatory effects, as in the case of leptin, TNF-α and IL-6 (Bugianesi and Pagotto 2005; Crespo 2001; Hui 2004; Maeda 2002; Xu 2003). In addition to being anti-inflammatory, adiponectin is also anti-lipogenic. Adipokines, involved in the metabolism of glucose and lipids both in the liver and in the periphery, may be present at altered levels in some diseases, such as in the case of NASH. In fact, studies show that NASH patients on the one hand have lower adiponectin levels, while on the other they have an increase in TNF-α levels (Bugianesi and Pagotto 2005; Hui 2004).

Role of adipose tissue

The accumulation of fat that affects NAFLD is defined as ectopic, being a condition in which, due to a lipid energy imbalance, there is an accumulation of lipids in non-traditional sites, such as the liver (Byrne and Targher 2014). The type of accumulation that occurs in the hepatic steatosis is responsible for the release of cytokines produced by the liver, and for other events such as the increased gluconeogenesis, the reduced synthesis of glycogen, and finally an inhibition of insulin signaling (Gao 2011; Kantartzis 2010; Samuel 2004). This hepatic condition also involves the onset of insulin resistance and an inflammatory stimulus, that lead to the progression of the pathology. Other events that favor this evolution in NAFLD patients are the increase in the expression of inflammatory genes and the macrophage activation in adipose tissue (du Plessis 2015).

Therefore, this dysbiosis affecting the adipose tissue is essential for the worsening of NAFLD disease (Jornayvaz 2012).

Adipose tissue releases various cytokines, such as IL-6 and TNF-α, which lead to a prolonged state of inflammation in obese patients(Fernandez-Real 2001; Makino 1998). Moreover, adipose tissue is an organ capable of performing endocrine functions, being able to release hormones such as leptin and adiponectin (Gregor and Hotamisligil 2007).

Leptin has the important role of preventing the accumulation of lipids in the sites where it should not occur. In obese patients, due to the presence of leptin-resistance, leptin levels increase promoting fibrogenesis (Ikejima 2001; Tsochatzis 2008). Furthermore, Kupffer cells stimulated by Leptin produce TGF-β1 and subsequently activate hepatic stellate cells via mTOR and hedgehog pathway, that in response to liver damage would lead to the formation of scar tissue (Aleffi 2011; Choi 2010; Wang 2009).

Adiponectin has both an anti-inflammatory, through the production of IL-10, and pro-inflammatory activity through the production and release of TNFα and interferon gamma (INF-γ), and a protective role in the liver (Tilg and Hotamisligil 2006). In fact, it seems to have a protective effect against insulin resistance, being an insulin-sensitizing hormone, it improves the oxidation of fatty acids, due to the activation of AMPK, and reduces gluconeogenesis. The acting effect of adiponectin appears to be mediated by its specific receptor, called AdipoR1, which is reduced in obese patients (Iwabu 2010). Furthermore, adiponectin, again thanks to the activation of AMPK (Adachi and Brenner 2008), would have antifibrotic effects (Kamada 2003).

Van der Poorten and colleagues analyzing the relationship between the regression of steatosis in patients with NASH and the levels of adiponectin, pointed out that an increase in the levels of this hormone would lead to an increase in the degree of hepatic steatosis, thus demonstrating the protective role of adiponectin in this disorder (van der Poorten 2013).

Intestinal microbiota

The intestinal microbiota is certain to play an important role in the development and evolution of NAFLD (Bugianesi 2016) . The bacterial population in the intestine produces active metabolites that are released into the splenic circulation. Half of the blood flow conveyed to the liver derives from the splenic circulation. Therefore, the liver is exposed to these bacterial products, which in part are also toxic.

One of the best known toxic compounds produced by bacteria is lipopolysaccharide (LPS), capable of activating the inflammatory cascade following TLR-4 binding: the pathways activated promote obesity, insulin resistance and the accumulation of fats in the hepatocyte (Noverr and Huffnagle 2004; Rivera 2007; Zeisel 1983). There are also enzymes produced by the intestinal microbiota, such as those capable of converting food-borne choline into toxic compounds, such as trimethylamine-N-oxidase (TMAO), a product capable of inducing the onset of an inflammatory state as well as damage hepatic (Zeisel 1983).

Furthermore, some bacterial types alter intestinal permeability with consequent release of bacterial products into the portal circulation (Mehal 2013). Variations in the abundance of intestinal microbiota

species, such as Bacteroidetes and Firmicutes, also cause an increase in energy uptake, a factor that promotes the onset of an inflammatory state and the onset of insulin resistance (Bugianesi 2016). In fact, it has been shown that NAFLD patients have greater intestinal bacterial growth and greater permeability in this organ than healthy subjects (Miele 2009). These changes are also observed in the case of NASH (Wigg 2001).

The energy effect is also due to the fact that the intestinal microbiota leads to the release, starting from compounds such as resistant starch, of short-chain fatty acids (SCFA), absorbable in the intestine (Topping and Clifton 2001).

Furthermore, the intestinal microbiota in diseases such as hepatic steatosis is capable of inducing de novo lipid synthesis in the liver and promoting the release of VLDL through an action on the farnesoid X receptor (FXR) (Tremaroli and Bäckhed 2012).

Another condition that can be observed in patients with NASH is the production of endogenous alcohol from the intestinal microflora, a condition that leads to an increase in blood alcohol levels, leading to a worsening of the clinical picture (Zhu 2013).

Genetic determinants

Researchers discovered the existence of genetic factors that can influence the risk of NAFLD, as well as the progression and development of the disease, and the results obtained through genome-wide association studies (GWAS) allowed the identification of polymorphisms found in some cases of NAFLD (Wang 2005).

Among the genetic variants that are associated with the development of NAFLD there is a single nucleotide polymorphism (SNP) involving the patatin-like phospholipase domain-containing 3 (PNPLA3) gene. In humans, this gene is found on chromosome 22 and encodes a protein, adiponutrin, of 481 amino acids weighing 53 KDa (Baulande 2001).

PNPLA3 shares significant homology with another member of the same family, the protein PNPLA2, also known as adipose triglyceride lipase (ATGL), which plays a key role in the metabolism of TG (Basantani 2011).

Furthermore, the progenitor of this family also plays the role of lipase as well as possessing the ability to cleave fatty acids from lipid membranes (Bánfalvi 1994; Shewry 2003).

Through studies conducted on insect cells they discovered, using a recombinant form of PNPLA3, that this protein has a lipase activity against triacylglycerols, and is capable of catalyzing the hydrolysis of monoacylglycerols, diacylglycerols and triacylglycerols (Huang 2011).

A single variant in PNPLA3 (*rs738409*) consists in the substitution of a cytosine in place of a guanine, which leads at the amino acid level to the substitution of an isoleucine with a methionine at position

148 (I148M) (Romeo 2008). This is recognized as the genetic variant most associated with both alcoholic and nonalcoholic steatosis (Romeo 2008; Singal 2014). As shown by in vitro studies, this variant leads to an 80% reduction in the hydrolytic activity against TG possessed by the protein (Huang 2011).

Studies in animal models show that this variant leads to an increase in the fat content in the liver, while it does not cause changes in glucose or lipoprotein metabolism (Minehira 2008; Monetti 2007). As for the mechanism by which this variant favors the accumulation of TG, there is no consistent evidence in favor of a mechanism mediated by a simple loss or acquisition of protein function. But a mutated form of the protein escapes from proteasomal degradation and tends to accumulate on the lipid droplets (LD) to a greater extent than the wild-type form (BasuRay 2017; He 2010).

PNPLA3 acts as a regulator of LDs, where it is associated with the endoplasmic reticulum and membranes (Chamoun 2013; He 2010; Pirazzi 2012). Furthermore, patients with NAFLD have an increase in de novo synthesis of lipids, which does not occur in patients with variant I148M (Mancina 2015), in whom a modest reduction in atherosclerosis was observed compared to the wild type form (Lauridsen 2018).

A hypothesis concerning the mechanism of action of the I148M form would be that at the level of the LDs it can prevent the hydrolysis of TG through a sequestration or a replacement of lipases or some of their cofactors (Wang 2019). This hypothesis is proposed and evaluated by Wang and colleagues, who discovered through in vitro studies how PNPLA3 protein, localized to LDs, can inhibit the hydrolysis promoted by ATGL lipase without altering its levels or affinity for LDs, but sequestering its CGI-58 cofactor and binding directly to it. Through this mechanism, mutated PNPLA3 can prevent the interaction of ATGL lipase on the surface of the LDs. Also mutated PNPLA3 leads to an increase in the size of the LDs highlighting how preventing the interaction between ATGL with its cofactor reduces the hydrolysis of TG (Wang 2019).

Other AA variants of the PNPLA3 protein have been identified, but only some of these are be associated with intra-hepatocyte lipid accumulation, such as the PNPLA3-S4531 form, which would be characterized by a lower accumulation of hepatic lipids (Romeo 2008).

Another gene associated with the progression of the disease, namely TM6SF2, located on chromosome 19 has been identified. In this case a SNP is involved in the establishment of NAFLD, *rs58542926*, which is associated with the loss of function of the coding protein, causing a reduction in the secretion of VLDL with consequent accumulation of TG in the hepatocytes, leading to the broad spectrum of NAFLD (Kozlitina 2014; Liu 2014).

9.4 NAFLD and Metabolic Syndrome

The term metabolic syndrome indicates a set of metabolic disorders, which generally occur in patients suffering from obesity, and which may be responsible for the establishment of or an outcome of insulin resistance (Yki-Järvinen 2014).
As defined by the National Cholesterol Educational Program (NCEP) Adult Treatment Panel III (ATP III) metabolic syndrome occurs if at least there are 3 of the following 5 criteria: circumference over 102 cm for men and 88 cm for women, blood pressure above 130/85 mmHg, fasting TG levels over 150 mg / dL, fasting HDL-C less than 40 mg/dL in men and 50 mg dL in women, and glucose levels above 100 mg/dL at fasting (Huang 2009).
Metabolic syndrome appears to manifest itself more in patients affected by obesity than in non-obese, although some non-obese subjects may be affected and other obese may not (Reaven 1988). Often patients with this syndrome have an excessive accumulation of TG in the liver as well as the insulin resistance of this site, thus causing NAFLD (Kotronen 2007).
Furthermore, in the liver the synthesis of two essential elements of the metabolic syndrome, namely VLDL, provide the greatest amount of TG, and fasting glucose. In fact, in presence of NAFLD there is an alteration in the ability of insulin to block the synthesis of these elements (Adiels 2007; Seppälä-Lindroos 2002). Once the liver has accumulated large quantities of TG, this produces elements such as CPR, fibrinogen and coagulation factors, that constitute CV risk factors (Anstee 2013; Targher 2009).
Metabolic syndrome and NAFLD often have overlapping characteristics, such as the range of disorders they can lead to, including NASH. In addition, both diseases can lead to the development of T2DM and CVD (Anstee 2013).
There is a linear relationship between the constituent factors of the metabolic syndrome and the hepatic fat content, measured by the most accurate diagnostic method of NAFLD, the proton magnetic resonance spectroscopy (1H-MRS) (Yki-Järvinen 2014). In the Rotterdam study of nearly 3000 patients, the prevalence of NAFLD was 35% (Koehler 2012). In these subjects the presence of some of the typical factors of the metabolic syndrome was found, such as the increase in waist circumference, the increase in fasting glucose levels and high TG levels (Koehler 2012). Furthermore, in the NHANES studies of more than 12,000 patients, the presence of insulin resistance increased the risk of NAFLD by 2.5 times (Lazo 2013).
Interestingly, cross-sectional studies have shown that components of the metabolic syndrome or the syndrome itself can be recognized as a risk factor for hepatic fibrosis and NASH. In particular, in one of these studies the metabolic syndrome was present more often in patients with NASH than in those

without (Brunt 2011). Two recent studies have also identified waist circumference, diabetes, hypertension and insulin resistance, typical of the metabolic syndrome, as predictors of NASH (Pais 2013; Sorrentino 2010).

9.5 NAFLD and CVD

Usually NAFLD patients manifest some typical elements of metabolic syndrome, as well as additional risk factors typical of CVD (Anstee 2013; Targher 2010). It is therefore not strange that NAFLD can increase the risk of developing CVD.
An association between NAFLD and different markers of subclinical atherosclerosis was highlighted in a meta-analysis conducted in 2013. These include the increase in the carotid mid-intimal thickness, the increase in aortic coronary calcification and the alteration of vasodilation mediated by flow and arterial stiffness (Oni 2013).
Cross-sectional studies also establish that CVDs are prevalent in NAFLD patients (Ballestri 2014; Bhatia 2012; Targher 2010), while retrospective studies underline that all causes of death, CVD mortality and liver mortality are higher in subjects affected by this form of steatosis, compared to healthy subjects (Dam-Larsen 2004; Ekstedt 2006, 2015; Söderberg 2010). In addition, some of these studies show that only in the presence of NASH, and not in presence of a simple NAFLD, there is an increase in mortality from all causes, as well as in CVD mortality (Ekstedt 2006; Söderberg 2010).
Further prospective studies show that NAFLD is associated with an increased risk of fatal and non fatal CVD events in individuals affected or not by diabetes mellitus (Stepanova and Younossi 2012; Targher 2007; Wong 2011; Zhou 2012). In a study published in 2011, Musso and colleagues found a double increased risk of fatal and non-fatal cardiovascular events in NAFLD patients (Musso 2011).
Attention should also be paid to the myocardium, as the presence of NAFLD is associated with metabolic defects in this organ (Ballestri 2014). In particular, the alteration of energy metabolism can cause an excessive accumulation of fat in the epicardial area, in the absence of ventricular alterations, and alter systolic and diastolic function (Perseghin 2008).
Patients with hepatic steatosis have increased insulin resistance and a reduced perfusion of the myocardium (Rijzewijk 2008). These myocardial disorders can arise early in NAFLD patients. There is a connection between NAFLD and cardiac dysfunctions in adult patients, but also in adolescent patients (Ballestri 2014). Therefore, there is a clear need for timely CVD risk assessment in patients diagnosed with NAFLD.

10. Mediterranean and Vegetarian diet in CVD

Diet has been considered an important factor in CVD since the 1950s, when an international study, The Seven Country Study, began. This study involved nearly 13,000 men in 7 different countries, and brought to light the protective role of the Mediterranean diet against cardiovascular diseases (Fung 2009; Keys 1986). Subjects who followed a high-fat diet, with a high ratio of mono-unsaturated fatty acid (MUFA) to saturated fatty acid (SFA), had a reduced risk of CHD after a follow-up of 25 years. (Menotti 1999). The Seven Country Study introduced the hypothesis of a possible relationship between eating habits and some chronic diseases.

Since then the role of diet has been the focus of much research. A variety of studies state that correcting eating habits and improving lifestyle, consisting mainly of exercising, not smoking and not abusing alcohol, leads to a reduction in cardiovascular morbidity and mortality (Dietary guidelines advisory committee reports 2015).

Therefore, a correct diet in terms of both quality and quantity is important for the prevention of CVD risk (Freeman 2017).

According to what has been reported by the American Heart Association (AHA), 7 factors impact CV health. Proper nutrition, as well as 4 other factors, connected with it, namely the BMI, blood pressure, total cholesterol and blood glucose, are included (Lloyd-Jones 2010).

Recent attention has been paid to some types of diet, in particular the Mediterranean and vegetarian ones. A common pattern of these diets is the preference of whole foods, fruits and vegetables, with reduced or no consumption of meats, especially red ones. These diets typically discourage the consumption of refined products. Given the typical foods used in, these diets are characterized by a high prevalence of fiber, vitamins and minerals, complex carbohydrates, polyunsaturated fats and phytochemicals (Cicero 2017).

The current guidelines for the prevention of CVD suggest adherence to healthy eating plans, which also benefit pro-inflammatory markers (Centritto 2009). These diets have elements in common such as the high intake of fiber, vitamins, antioxidants, minerals, polyphenols, MUFA and polyunsaturated fatty acids (PUFA). In addition, they are characterized by a reduced intake of elements that could have a negative impact on health, such as salt, sugars, especially refined ones, saturated and trans fatty acids (TFA) (Mozaffarian 2016). Preferred foods are fruits, vegetables, legumes, fish and seafood, nuts and seeds, whole grains, vegetable oils and dairy products, while foods to be consumed less frequently are confectionery products, red meat and processed meat (Mozaffarian 2016; Silveira 2018).

The Mediterranean diet represents a typical food style of the populations of the Mediterranean basin in the 50's and 60's. This dietary model is defined by the preferred foods, in particular the high consumption of fruit, vegetables, legumes, cereals, fish, dairy products, the modest consumption of ethanol, especially as wine and the reduced consumption of meat, especially the red met. Another important factor is the high ratio of MUFA to SFA fats (Trichopoulou 2003; Trichopoulou and Lagiou 2001).

It is clear that this diet does not provide for calorie restrictions, but rather suggests the consumption of foods which, if taken together, provide protection against different types of disorders (Martinez-Gonzalez and Martin-Calvo 2016), consistent with what is suggested by the AHA (Kris-Etherton 2007; Lichtenstein 2006).

Many studies on the benefits of the diet have been conducted over the years. One of the best known secondary prevention studies is the Lyon Diet Heart Study, which is a randomized controlled trial that tests the effect of adherence to a Mediterranean diet on the recurrence of coronary events in patients who have already had a heart attack. After 46 months, patients subjected to the Mediterranean-type diet show a reduced risk of relapse between 30 and 70% (de Lorgeril 1999). More recent, however, is the PREDIMED study, a primary prevention multicenter study, which involved more than 7,000 people with high cardiovascular risk. The people involved in this study were subjected to one of the two types of Mediterranean diet, one enriched with extra virgin olive oil, and the other enriched with a mix of nuts, or to a low-fat diet (control group). After 4.8 years of adherence, the two types of Mediterranean diets resulted in a significant reduction (approximately 30%) in the risk of heart attack compared to the control group, without no difference between the two types of Mediterranean diets (Estruch 2018). In a sub-study of the PREDIMED study, Estruch and colleagues demonstrated that adherence to the Mediterranean diet also resulted in benefits on inflammatory markers. In particular, patients subjected to a diet enriched with extra virgin olive oil demonstrated a reduction in CRP, while in both groups adhering to the Mediterranean diet there was a decrease in IL-6, ICAM-1 and VCAM-1(Estruch 2006). Regarding the other cardiovascular risk factors, in this study there was a reduction in systolic and diastolic blood pressure, in glucose levels, in the cholesterol-HDL-C ratio, fasting insulin levels and an increase in HDL-C levels (Estruch 2006). This shows the effects of the Mediterranean diet on the lipid profile, blood pressure, insulin resistance, as well as on pro-inflammatory markers (Casas 2017; Estruch 2006; Martínez-González 2015). Therefore, since the atherosclerotic plaque is determined in part by inflammatory processes, the Mediterranean diet could also have an impact on the formation and development of atheroma. Further studies also demonstrate a benefit of this diet on metabolic syndrome and diabetes (Salas-Salvadó 2008, 2011).

A 2015 study by De Filippis and colleagues demonstrates how correct adherence to the Mediterranean diet also has a positive impact on the intestinal microbiota. The diet lead to an increase in SCFA in faeces, due to a change in the quantity of microbial species belonging to species such as Firmicutes and Bacterioides, capable of degrading those carbohydrates, not normally metabolized by humans (De Filippis 2016).
In addition, a meta-analysis study conducted by Grosso and collaborators, showed that high adherence to the Mediterranean diet reducing the risk of stroke by 24%, coronary heart disease by 28% and myocardial infarction by 33% (Grosso 2017). Another meta-analysis study supporting the relationship between adherence to this diet and cardiovascular outcomes was published by Dinu and colleagues. This study showed that adherence to the Mediterranean diet reduced the risk of incidence of cardiovascular events and mortality, reduced BMI and inflammatory biomarkers, and also improved the lipid and glycemic profile (Dinu 2018). Further studies show that the Mediterranean diet improves the composition and oxidative state of HDLs (Hernaez 2017).
The Mediterranean diet would therefore affect several aspects of the cardiovascular field. It promotes the reduction of cholesterol, especially in the form of LDL, and have a protective effect against inflammation and oxidative stress. The latter effect, in particular, would be promoted in part thanks to the presence of antioxidants vitamins, as well as phytochemicals, minerals and folates in the diet (Mentella 2019).
Finally, the Mediterranean diet modifies the bacterial intestinal flora, increasing the bacterial species, named Bacteroides, and reducing the levels of the Firmicutes species, leading to a reduction of pro-atherogenic products, that originate from diet-derived elements, such as TMAO (Merra 2020).
The vegetarian diet that has aroused great interest in recent years (Leitzmann 2014), given the evidence that reducing the consumption of animal products, reduces CV risk (Salter 2013).
The salient feature of this type of diet, which would also be responsible for the positive outcomes on human health, such as the reduction of CVD risk, would be the high consumption of plant products, such as fruit, legumes, untreated cereals and nuts. In addition, the presence of phytochemicals with antioxidant power is important (Freeman 2017).
People who follow this type of diet show reduced levels of total cholesterol, LDL-C, blood glucose and blood pressure, as well as reduced in insulin resistance and lower BMI levels (Dinu 2017). All these factors seem to have a cardiovascular benefit, but there is limited evidence supporting these.
AMS study conducted by Key and colleagues focused on members of the Seven-day Adventist church, who, in addition to following a vegetarian diet, consume little amount of alcohol and are not habitual smokers. The mortality rate from cardiovascular and cerebrovascular diseases was significantly lower than in subjects who consumed meat (Key 1999). In another study subjects who

followed a vegetarian diet had a 38% reduction in mortality from heart disease, compared to the population who consumed an omnivore-type diet, who also had increased obesity, hypertension as well as T2DM (Fraser 1999). Another study, currently still in progress, would also seem to support the vegetarian diet reduces of traditional risk factors and reduced the risk of CV events in male patients by 29% (Orlich and Fraser 2014).

Finally, the EPIC study showed a reduction by 32% in the risk of heart disease in patients who followed a vegetarian diet compared to subjects who followed an omnivorous diet, after an 11-years follow up. This is promoted thanks to the beneficial effects on cholesterol and blood pressure levels (Crowe 2013).

Despite this, in a re-analysis of the data resulting from this latest study, scientists demonstrate the lack of a significant difference in cardiovascular mortality rates between patients who followed a vegetarian diet and an omnivorous diet (Appleby 2016). In a recent study, Sofi and collaborators compared the effects of the Mediterranean diet and a type of vegetarian diet, called lacto-ovo vegetarian (VD), on body weight and CV risk (Sofi 2018). This recent developed form of vegetarian diet is characterized by the exclusion of all types of meat, while allowing the consumption of eggs and dairy products (Leitzmann 2014). Both of these types of diets promote the reduction, without differences in body weight, fat mass or BMI; However, VD was more effective in reducing LDL, while the Mediterranean diet was more effective in reducing TG (Sofi 2018).

Part I: Role of Lp(a) genetic variants and cholesterol loading capacity in patients with elevated Lp(a) and coronary heart disease submitted or not to lipoprotein apheresis

Aim

Studies report that the increase in Lp(a) concentration leads to an increment in the risk of coronary heart disease (CAD) (Danesh 2000; Sharrett 2001; Stulnig 2019). In a 2011 study, an association was observed between the small size of Apo(a) and the increased risk of CAD (Lamon-Fava 2011), while in another study it was shown that the small size of this component is associated with a higher concentration of Lp(a) (Wu 2004); moreover in a further analysis it was seen that the size of Apo(a) is a better predictor for CAD risk than the concentration of Lp(a) (Rifai 2004).
To date, the best and only effective therapy in patients with high levels of Lp(a) and CAD is lipoprotein apheresis (LA). The ability of serum to load macrophage cells with cholesterol is a pro-atherogenic ability, which is evaluated through a parameter defined as cholesterol loading capacity (CLC). This parameter indicates not only the quantity of free cholesterol that flows into cells (Weibel 2014), but also the quantity of cholesterol that escapes from macrophages thanks to plasma lipoproteins (Zimetti 2006). An index for the evaluation of pro-atherogeneity at the cellular level therefore consists in the increase in CLC, that leads to the formation of foam cells. In particular, this index is increased in CV diseases, as well as being associated with an increase in CV risk (Adorni 2012; Ronda 2015).
Located on chromosome 6q27, the gene encoding Lp(a), known as the LPA gene, has a high sequence homology, between 78 and 100%, with human plasminogen at the level of the untranslated and coding sequences (Schmidt 2016).
A characterizing element of Apo (a) are the Kringles domains, which are triple ring structures, stabilized by the presence of 3 internal disulfide bridges. Of particular importance is the kringle IV domain, referred to as KIV, which in Lp(a) is present in 10 different types, each of which has a unique amino acid composition. Among these domains some are present in single copy, ie KIV_1 and from KIV_3 to KIV_{10}, while KIV_2 has a variable number of copies, ranging from 1 to 40 sequence repetitions. The latter phenomenon is known as the polymorphism of the variation in the number of copies of KIV_2, a predictor factor of 40/70% of the variations in plasma levels of Lp(a) (Enkhmaa 2011).
Two genetic variants, *rs3798220*, a SNPs in the protease domain of a single nonsynonymous nucleotide, and *rs10455872*, an intron SNP, were found in LPA, both associated with elevated Lp(a) levels, as well as a high CV risk (Clarke 2009). In addition, seven other SNPs were discovered thanks to linear regression studies, each of which was associated with a statistically significant increase in the concentration of Lp(a), and are responsible for 40% of this variation.
The high association between CAD and each of the two SNPs, *rs10455872* and *rs3798220,* has been demonstrated through genome-wide studies (Lu 2015; Mack 2017).

An increase in Lp(a) levels were found both in the presence of familial hypercholesterolemia (FH) (Utermann 1989) and in the case of familial defective Apo B-100 (van der Hoek 1997). Furthermore, other genes were found to be involved in the metabolism of this lipoprotein, namely APOE and PCSK9.

APOE2 appears to be an isoform associated with a 15% reduction in Lp(a) levels (Mack 2017), as well as the loss of function (LOF) R46L mutation of PCSK9 (*rs11591147*) would reduce the concentration of Lp(a) (Erhart 2018).

In this multicentric Italian study on Lp (a), promoted by Mighty Medic, were involved 3 clinical centers and 2 laboratory centers, including the laboratory of professor Livia Pisciotta and the laboratory of professor Elda Favari. The aim was to analyze the relationship between Lp(a) and a serum pro-atherogeneity index at the cellular level, named CLC, by determining the levels of Lp(a) as well as the size of Apo(a) in patients affected by CAD and with a high concentration of Lp(a). In addition, in these patients the intent was to evaluate the impact of LA on the concentration and size of the lipoprotein and on the CLC levels. Finally, gene sequencing was performed for polymorphisms involving LPA gene on patients with high amounts of Lp(a).

Materials and methods

Patients

This observational study involves patients from multiple centers, affected by hyperLp(a), in isolated and combined form, as well as by CVD.

34 patients were involved, of which the average age was 59.5 ± 8.9 years and 70% of which were males. The patients came from three different Italian hospitals, of which 24/34 came from Rome (prof. Claudia Stefanutti), 9/34 from Verona (Dr Maria Grazia Zenti) and finally 1/34 from Pistoia (Dr Daniele Berretti).

All 34 patients were on lipid-lowering therapy, 14 of which were on statin therapy, 16 on statin and ezetimibe therapy, 1 patient on alirocumab and finally 1 on lomitapide.

In addition, 18 of the patients were on apheresis therapy, referred to as the LA group, while 16 with HyperLp (a) and CVD had not undergone this additional procedure.

In the results session, in table Ia, the characteristics of the patients are reported; finally, the 18 patients in the LA group underwent long-term extracorporeal therapy for an average of 5 years (range 1-27 years).

Apo(a) isoform size evaluation

Between the chemicals used there were Ammonium bicarbonate (ABC), dithiothreitol, iodoacetamide, sodium deoxycholate (DOC), and formic acid, all supplied by Sigma-Aldrich Srl. Mass Spectrometry Grade Trypsin Gold (code number: V5280) was supplied by Promega Srl (Promega, Milan, Italy) and performed by Professor Vacondio at the Department of Food and Drug, University of Parma. To determine Apo (a) concentration was used Proteotypic peptide LFLEPTQADIALLK, while for KIV-2 repeating sequence was used GTYSTTVTGR, and the corresponding standards labeled with $^{13}C^{15}N$, from New England Peptides (Gardner, MA). Prof. Eduardo Angles-Cano offered the calibrators for recombinant apo(a) isoform to detect apo(a) size, that contain respectively 10, 14, and 18 kringles.

Human serum samples: Tryptic digestion

In order to proceed with the digestion of human serum samples, carried out by Professor Vacondio, the protocol already available (Lassman 2014) was used, with the addition of small changes.

To reach the final volume of 200 μl, 4 μl of the plasma samples of the patients under study were diluted in 132 μl of ABC 50 mM at pH 8.0, as well as 10 μl of the 10% w/v DOC solution and finally 50 μl of a 100 nM solution of labled internal standards in ABC. At this point the samples are reduced-alkylated-digested following a standard protocol by means of 2 μL of a solution of dithiothreitol at a

concentration of 500 mM for the duration of 30 minutes at a temperature of 60 °C. Subsequently, 2 µL of a solution of iodoacetamide at a concentration of 1 M were added for 1 hour in the dark, at room temperature. Digestion was then performed overnight with 3 µg of trypsin for each sample. 10 µL of 20% v/v formic acid was used to block the digestion process and to favor the precipitation of DOC.

For 15 minutes at 3000 rpm at 4 ° C the samples were centrifuged and finally 10 µL of the acid supernatant were inserted for analysis in the High-performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS).

Tryptic digestion of recombinant Apo (a) isoforms

The calibrators used are three recombinant Apo (a) isoforms, in which the stock solution was supplied in culture medium enriched with 10% fetal bovine serum, coming from a CELLine bioreactor. The concentration already determined, at the time of delivery, was carried out by the IED rocket electrophoresis assay, and corresponded to 0.948 mg/ml for 10 kringles of Apo (a), while for kringles 14 it was 0.339 mg/ml, and for the 18 kringles was 0.675 mg/ml. Following the serial dilution, the stock solutions of the recombinant Apo (a) were subjected to digestion with trypsin in the presence of human serum (4 µL) not containing appreciable levels of the proteotypic peptides, as they were previously digested, following the same protocol already outlined.

HPLC-MS/MS analytical method

Using the Thermo Accela® UHPLC system, united to a Thermo TSQ Quantum Access Triple Quadrupole Max Mass Spectrometer (Thermo, Milan, Italy), equipped with a heated electrospray ionization (HESI) ion source, it was possible to analyze the previously digested samples, performed by Professor Vacondio. The samples were separated by gradient elution. Acetonitrile and 0.1% formic acid were used as eluent A, while water and 0.1% formic acid were used as eluent B.

The gradient was set as follows: t = 0 minutes: 2% A; 98% B; t = 5 minutes: 2% A; 98% B; t = 20 minutes: 30% A; 70% B; t = 22 minutes: 95% A; 5% B; t = 23 minutes: 95% A; 5% B; t = 24 minutes: 2% A; 98% B followed by 4 minutes of rebalancing of the chromatographic column. The total gradient time was 28 minutes.

A Phenomenex Synergi Fusion C^{18} column (150x2.1 mm, 3 µm particle size) and an HPLC flow of 350 µl/ min was used. The injected volume was 10 µL. The acquisition by the mass spectrometer took place in positive electrospray ionization mode (ESI +), monitoring the transitions between the molecular ion and the product ions of the tryptic peptides analyzed (Multiple Reaction Monitoring or

MRM mode). The electrospray ionization of the peptides was optimized by direct source injection (Flow Injection Analysis or FIA) of a 10 μM solution of each analyte dissolved in a solution containing 50% water + 0.1% formic acid: 50% methanol.
The following transitions between parent ion and product ion were monitored: GTYSTTVTGR [M + 2H]$^{2+}$ m / z = 521.8→721.4; 634.5; 136.0 (Tube Lens (TL) = 74V; Collision Energy (CE) = 19; 20; 27 eV); Internal standard GTYSTTVTGR [$^{13}C_6{}^{15}N_4$] [M + 2H]$^{2+}$ m / z = 526.8→ 731.4; 543.2; 135.9 (TL = 75 V; CE = 19, 20, 28 eV); LFLEPTQADIALLK [M + 2H]$^{2+}$ m / z = 786.5→1070.0; 260.8; 232.9 (TL = 101 V; CE = 27; 31; 38 eV); Internal standard LFLEPTQADIALLK [[$^{13}C_6{}^{15}N_2$] [M + 2H]$^{2+}$ m / z = 790.5→1078.0; 260.8; 232.8 (TL = 101 V; CE = 26; 30; 36 eV).
Thermo Xcalibur software, version 1.3, was used for the acquisition and processing of HPLC-MS / MS data. GraphPad Prism version 6.01 was used for data analysis and graphs.

Cell line

Kept in sterile conditions, cell cultures were manipulated with a vertical laminar flow hood validated for the use of toxic and carcinogenic substances. Cell cultures are kept in an incubator (RS Biotech, UK) at 37 ° C, at 5% CO2 and with a humidity of 90-95%. The morphological observation of the cells was carried out with an inverted optics microscope (Leica Microsystems, Germany).
For CLC protocol was used a line of human monocytes, THP-I. This cell line grows in suspension in RPMI 1640 (Thermo Fisher Scientific) containing HEPES, Sodium Pyruvate 1mM and glucose 2.5mg/ml (Sigma-Aldrich), gentamicin 50 mg/ml, ß-mercaptoethanol 50 mM (Thermo Fisher Scientific), enriched at the time of use with 10% FBS. For the subcultivation passages THP-I are maintained in RPMI 1640 at 10% FCS.
For seeding, the cells are differentiated to macrophages by treatment with phorbol myristate acetate (PMA, Sigma-Aldrich) at a concentration of 100 ng/mL.

Evaluation of Serum-Mediated Macrophage CLC

To evaluate CLC were used THP-1 cells, sown in 24-well plates at a density of 500000 cells per well, in a medium added with 10% of FBS and PMA (100 ng/mL). After 72 hours the differentiation into macrophages has taken place, so cells are incubated with 5% (v/v) of patients' serum, who act as cholesterol donors, for 8 hours.
At the end, after a wash in PBS, macrophages are lysed in a solution of 1% cholic acid and DNA-asi (50 U/mL) and left to stir overnight. CLC is evaluated as μg of cholesterol per mg of protein.

Amplex Red Cholesterol Assay Kit (Molecular Probes by Life Technologies), which is a fluorimetric kit, is used for the quantification of the cholesterol content in the lysates, while to evaluate the protein content in the lysates was used the bicinconinic acid method.

Statistical analysis

Mean ± SEM was calculated to express the results. The differences for the variables referring to the lipid profile before and after LA were analyzed by paired t-test and were reported in Table Id. The Bonferroni correction was used as multiple comparisons were made, and it was applied as a statistical cutoff to each comparison set to 0.007 (α/n, α: p-value and n: number of comparisons). In addition, two nested linear regression models were used to evaluate the effects of different variables on CLC levels. The first of these models involved variables such as age and sex, as well as smoking, BMI, LDL-C, Lp (a) and TG levels, in which the latter are explanatory. On the other hand, categorical variables are gender and smoking, in which the categories taken as reference are respectively male and non-smokers. The second model, on the other hand, provides for the addition of a further variable, namely Lp(a) size (Kn). The F test of statistical significance on the variation of R^2 was used to evaluate the melioration in the model fit between the two nested models.
A limited number of variables were used for model regression due to the small number of patients. Total cholesterol was not considered due to the collinearity with TG and LDL, while the exclusion of HDL is due to the fact that they have no biological importance for the accumulation of cholesterol in the cell.

Results

Study patients

Table Ia shows the demographic parameters, pathologies and pharmacological and non-pharmacological treatments, while Table Ib shows the lipid parameters and the basal CLC values. In both tables the patients were divided into two groups, of which the first included patients undergoing lipid lowering therapy and LA, while the second included patients who underwent lipid lowering therapy, but not LA. Between the two groups, similar variables were arterial disease, smoking habits and basic lipid profile, on the contrary in the first group, i.e. for patients who underwent LA, CAD was more severe and also CLC was greater than in the control group (19.6 ± 3.7 µg/mg vs 15.7 ± 3.5 µg/mg of protein, P = 0.004).

	Pharmacological Treatment + LA (N=18)	Pharmacological Treatment (N=16)	P *
Sex (F/M)	2/16	8/8	
Age (years)	60±10	59±8	Ns
BMI (Kg/m^2)	26.0±3.0	24.8±2.0	Ns
CAD (# vessel involved) (mono-/bi-/trivasal) N	4/6/6	2/4/2	Ns
Impaired epiaortic arterial axes(stenosis<69%/CAS o CEA) N	3/3	2	Ns
Cigarette smoking (previous, never or current) N	11/7/-	37504	Ns
Lipid-Lowering therapy (Statins/statins+ezetimibe/PCSK9I /lomitapide) N	5/10/1/1	9/6/-/-	Ns

Table Ia: patients' demographics, diagnosed pathologies and pharmacological treatment. CAD=coronary artery disease, CAS=carotid angioplasty and stenting, CEA= carotid endarterectomy, LA=lipoprotein apheresis. Variables are presented as mean ± SD. *indicates P-value

	Pharmacological Treatment + LA (N=18)	Pharmacological Treatment (N=16)	P *
Total cholesterol (mg/dL)	171± 39	180±39	Ns
LDL cholesterol (mg/dL)	97±34	106±34	Ns
HDL cholesterol (mg/dL)	50±10	50±8	Ns
TG (mg/dL)	123±56	121±34	Ns
Lp(a) (mg/dL)- median	117	115	Ns
[range i.q.]	[94,3-135]	[99,2-137,7]	
CLC µg/mg of protein	19,7±3,7	16,1±3,57	0,009

Table Ib: Lipids, lipoproteins, and CLC at baseline. Variables are presented as mean ± SD, exept for Lp(a) expressed as median. CLC=cholesterol loading capacity, Lp(a)=lipoprotein (a). *indicates P-value

Comparison of MS-based and Ab-based methods for determination of serum levels of Lp(a)

For this multicenter study the analysis of human sera samples (n = 52) was conduced via HPLC-MS / MS analytical method, performed by Professor Vacondio. The tryptic peptide LFLEPTQADIALLK (LFLE) is unique and representative of Apo (a), as demonstrated by Lassman (Lassman 2014). It is

present only in the constant portion of Apo (a), therefore, after tryptic digestion, the area of the LFLE peptide signal turns out to be proportional to the molar concentration of Apo (a). Its internal isotope-labeled LFLE * standard, eluting at the same time as retention during chromatographic elution in HPLC, can be used to normalize signal suppression or augmentation effects of the corresponding ion due to co-eluting serum components with samples.

The relationship between the areas of the LFLE peptide and its internal standard LFLE * were correlated with the levels of Lp (a) (mg/dL), assessed using the antibody-based immunoturbidimetric method (Ab-based) (Koivunen and Krogsrud 2006).

A positive monotonic correlation was observed between the two variables in question with P <0.0001, as can be seen in Figure Ia from Spearman's non-parametric correlation with $\rho s = 0.8315$.

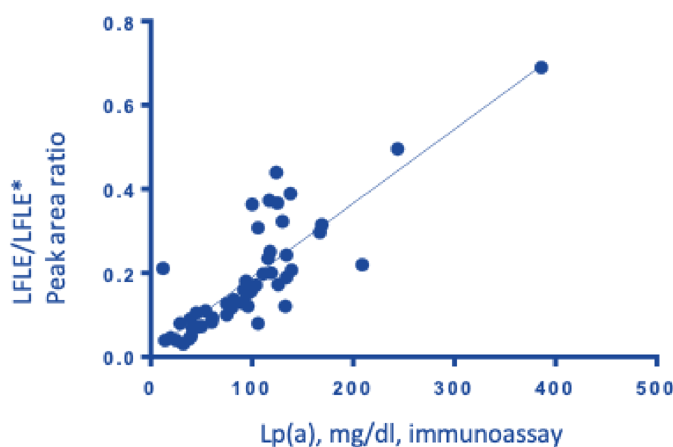


Figure Ia: Correlation between MS- and Ab-based apo(a) and Lp(a) serum quantification. Ab= antibody; MS= mass spectrometry.

Apo (a) average size

In order to relate the ratio of the peptide areas to the average size of Apo (a), digestion of 3 recombinant isoforms of Apo (a), which contained a different number of copies of KIV2 (K10, K14 and K18), was used.

As it can be seen in Table Ic, the ratio between the areas of the two GTYS/LFLE peptides, in fact, remained constantly as the concentrations of the recombinant proteins used increased, while it proportionally increased in the function of the copies of the KIV_2. The relationship between the ratio of the GTYS/LFLE peptide areas and the number of kringle present in the recombinant Apo(a) is used to: calculate the mean number of KIV-2 in the patient samples of the study; to correlate the mean size of the Apo (a) to the CLC in vitro; to evaluate the effect of apheretic, pharmacological and combined treatment on the size distribution of the Apo (a).

K10 Apo(a) concentration (nM)	Ratio GTYS/LFLE	Average Ratio	SD	RSD%
2.7	0.96	0.98	0.09	8.94
5.3	0.87			
14	1.00			
27	1.08			
K14 Apo(a) concentration (nM)	**Ratio GTYS/LFLE**			
3.55	2.69	3.26	0.80	24.60
7.1	2.91			
18	2.63			
35	4.80			
355	3.30			
710	3.25			
K18 Apo(a) concentration nM	**Ratio GTYS/LFLE**			
3.2	6.56	5.53	0.72	13.09
6.4	5.30			
16	4.87			
32	5.38			

Table Ic: Relation between KIV-2 copy number in recombinant Apo(a)s and GTYS/LFLE ratio at different protein final concentrations

Impact of LA on plasma lipids

Table Id shows the lipid values before and after treatment with LA.

Reduced lipid levels after LA treatment were: TC [-51.64% (P = 0.00001)], TG [-52.03% (P = 0.00001)], LDL [-69.67 % (P = 0.00001)] and Lp (a) [-65.43% (P = 0.00001)]. HDL levels are also reduced [-15.69% (P = 0.0003)].

To analyze the effectiveness of the LA treatment in allowing the reduction of the Lp (a) concentration, the HPLC-MS/MS method was used, through the evaluation of the percentage decrease in the ratios of the LFLE / LFLE * peptide area in patient samples before and after apheresis as shown in Figure Ib. The average percentage of the decrease was 66.2 ± 11.7%, not different from what was obtained with the second method, that is the Ab-based one, where it was 65.0 ± 11.7%; only in 4 patients the difference in the percentage of decrease between the two methods was greater than 15%.

Lipid parameter	Before lipoprotein Apheresis	After lipoprotein Apheresis	t	P-value
TC (mg/dl)	171,2 ± 9,2	82,8 ± 4,7	**11,452**	**<0.001**
TG (mg/dl)	123,4 ± 13,1	59,2 ± 9,8	**8,116**	**<0.001**
LDL-C (mg/dl)	97,6 ± 7,9	29,6 ± 3,0	**9,478**	**<0.001**
HDL-C (mg/dl)	49,7 ± 2,3	41,9 ± 2,0	**3,677**	**<0.001**
Lp(a) (mg/dl)	131,6 ± 17,9	45,5 ± 6,3	**6,639**	**<0.001**
CLC (mg/dl)	19,7 ± 0,9	16,1 ± 0,8	**3,139**	**0.003**
Apo(a) size (average KIV2 copy number)	19,8 ± 0,7	18,1 ± 0,5	2,646	0,009

Table Id: Paired t-tests for a set of variables before and after lipoprotein apheresis. CLC= cholesterol loading capacity; TC= total cholesterol; TG= triglyceride; LDL-C= LDL cholesterol; HDL-C= HDL cholesterol; KIV2= kringle IV type 2; Lp(a)= lipoprotein (a). Values are reported as mean ± SEM with n=18. Variables presented in bold are statistically significant at P<0.007, after Bonferroni's correction for 7 comparisons.

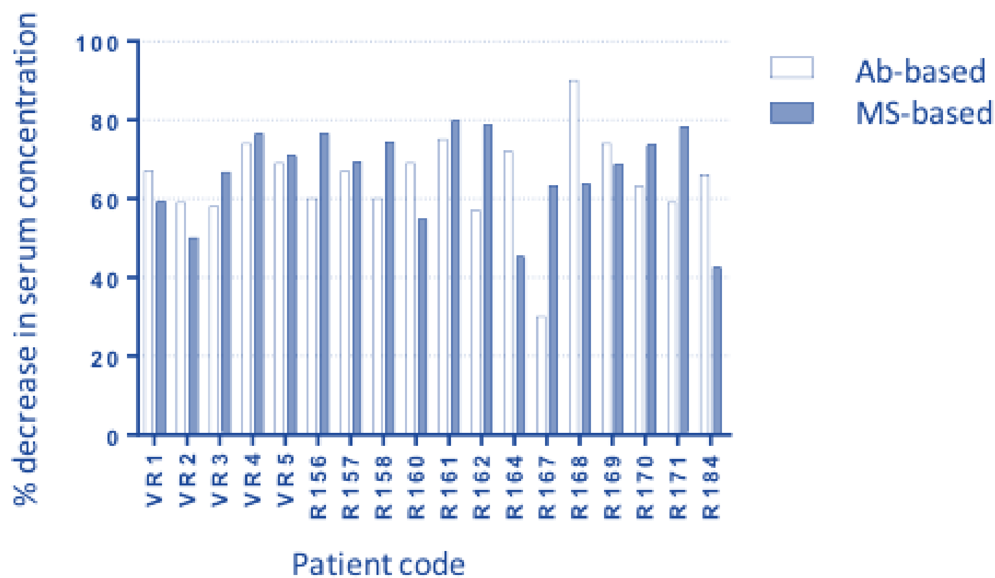


Figure Ib: Ab- and MS-based methods comparison for the analysis of the LA effect of on Lp(a) concentrations. Ab= antibody; Lp(a)= lipoprotein(a); LA= lipoprotein apheresis; MS= mass spectrometry.

Patient serum macrophage CLC before and after lipoprotein apheresis

To evaluate the serum CLC of patients before and after treatment with LA, THP-1 macrophage cell model was used. As shown in Figure Ic the amount of cholesterol at the cellular level before serum exposure was 13.90 ± 0.54 µg / mg. The serum CLC of pre apheresis patients (mean ± SD, 19.7 ± 0.9 µg / mg protein) was higher than that of normolipidemic serum, considered as control (mean ± SD, 16.01 ± 0.98 µg / mg of protein), while following apheresis treatment the CLC of study patients was reduced compared to sera before apheresis (mean ± SD, 16.1 ± 0.8 µg / mg of protein; P = 0.003) and comparable to that of the control serum.

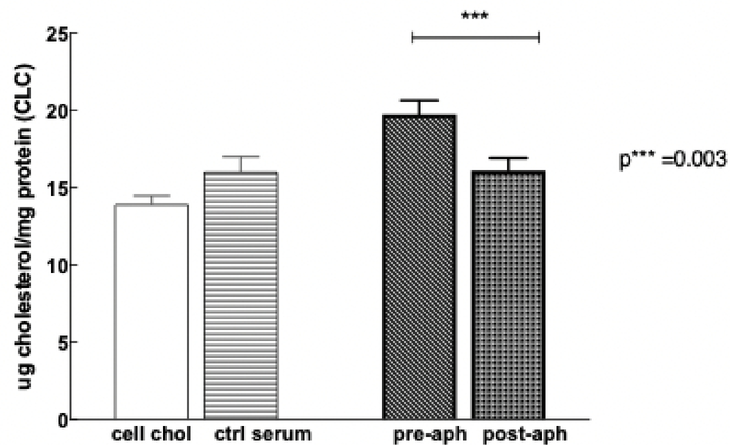


Figure Ic: comparison of the CLC of the different groups. In figure is represented in the first two columns cellular cholesterol content before serum exposure and after serum exposure, while in the latter two columns are represented CLC of pre-apheresis and post-apheresis patients. Post apheresis patients shows a lower CLC than pre apheresis patients (p=0,003), similar to a control serum. Data are shown as mean ± SD.

As can be seen from Figure Id, apheresis did not significantly affect the distribution of the size of the Apo (a) isoform, evaluated through the average of the ratios of the GTYS/LFLE peptide area and the number of copies of KIV2.

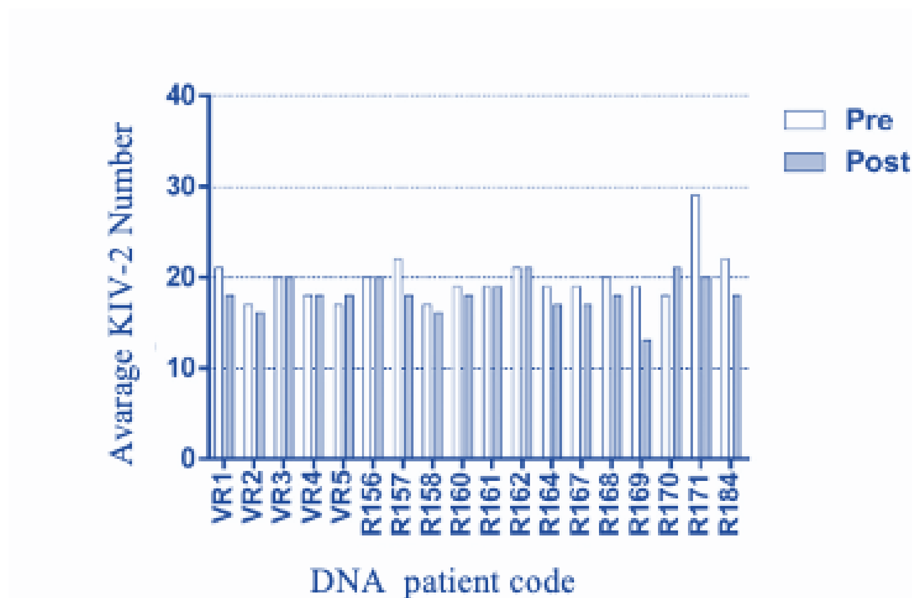


Figure Id: Comparison of the LA effect on Apo(a) isoform distribution, evaluated by changes in the GTYS/LFLE peptide area ratio. KIV2= kringle IV type 2; LA= lipoprotein apheresis.

Serum CLC and Lp(a) concentration and size

Figures Ie and If show through univariate analyzes in the first case a weak positive association between CLC and the concentration of Lp (a) (ρ = 0.094, P = 0.596), while in the second case a moderate negative association between CLC and dimensions of Lp (a) (ρ = -0.637, P <0.001).

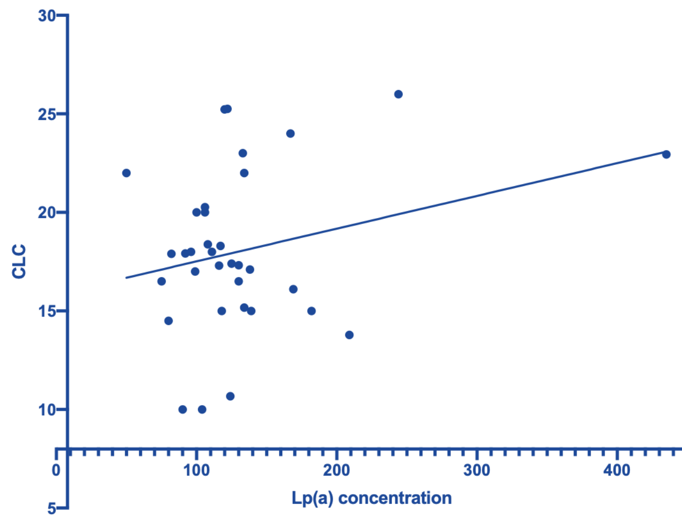


Figure Ie: Scatterplot of the relationship between CLC and Lp(a) concentration.

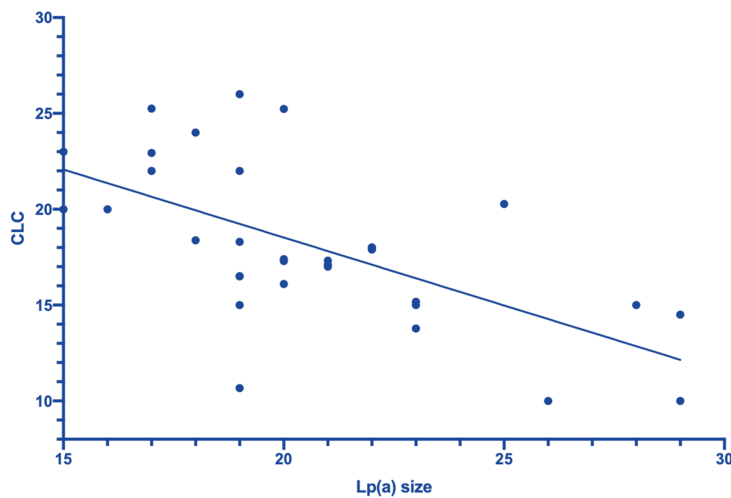


Figure If: Scatterplot of the relationship between CLC and Lp(a).

When analyzed by multiple linear regression for various confounding factors as shown in Table Ie, in the first model, Lp (a) correlated significantly with CLC (P = 0.035), while the other parameters were not.

Although in general the linear model does not adequately describe the experimental parameters (F = 2.1, P = 0.088). Instead, in the second model, adding the dimensions of Lp (a), the number of copies of KIV2, allows us to underline the inverse relationship with the CLC (β = -0.707, P = 0.001).

This second model, moreover, ensures greater robustness at the statistical level of the data (F = 4.3, P = 0.003).

	Model 1			Model 2		
Variables	β	P-value	Partial R^2	β	P-value	Partial R^2
Age (years)	0,013	0,875	0,001	0,007	0,907	<0,001
BMI (kg/cm^2)	-0,247	0,314	0,029	0,007	0,98	<0,002
Sex	-3,06	0,176	0,095	-2,435	0,263	0,104
Nonsmoker (ref. cat. smoker)	0,304	0,869	0,001	0,172	0,892	0,001
LDL-C (mg/dL)	-0,017	0,555	0,024	-0,013	0,561	0,02
Lp(a) (mg/dL)	0,023	0,035	0,106	0,012	0,412	0,051
Triglycerides (mg/dL)	0,015	0,463	0,03	0,02	0,163	0,093
apo(a) size (average KIV-2 copy number)				0,707	0,001	0,429
F (7, 22)	2,1			4,3		
P-value	0,088			0,003		
R^2	0,252			0,573		

Table Ie: Linear regression of CLC on a set of covariates: nested models. β=regression coefficient. CLC= cholesterol loading capacity; LDL-C= LDL cholesterol; Lp(a)= lipoprotein (a).

The F test on the variance of R^2, shown in Tables If, allows to highlight the statistical significance of the improvement between the two models.
The parameter in fact increases by 0.321 from model 1 to model 2, and the partial R^2 of the size of the lipoprotein in model number 2 is 0.429, thus explaining that the 43% of the overall variability of the CLC is due to the size of the LP (a), once checked for other variables under study.

	Block residual					
Block	F	df	Df	P-value	R2	change in R2
1	2,1	7	22	0,088	0,252	
2	16,2	1	21	0,001	0,573	0,321

Table If: Amelioration in model fit between the 2 nested models, F test on the change in R2.

Discussion

In this study, as reported in Table Ib, it was shown that patients, who have high levels of Lp(a), as well as an increased risk of CAD, and who have undergone apheresis, have high levels of CLC.
It was also seen that apheresis was a procedure capable of reducing the levels of Lp(a) and also of CLC, both of which were statistically significant. On the contrary this method did not change the size of the Apo (a) isoform.
Apheresis allowed to lower serum lipid levels and, moreover, by HPLC MS / MS it was possible to evaluate the parameters related to both Apo (a) and Lp (a).
Similar to LDL, Lp (a) is a particle with a hydrophobic core, containing Apo B-100, which ,through a disulfide bridge, is bound to another glycoprotein component, called Apo (a) (Furbee and Fless 1996), of which now at least 34 isoforms are recognized (Marcovina 1993). The number of structural subunits, named Kringles, and especially the number of copies of KIV2, that can vary from 3 to more than 40 copies, is an important determinant of the size of Apo (a).
Normally the methods for quantifying Lp (a) in mg/dL are Ab-based and are tests that do not take into account either the characteristic heterogeneity of the lipoprotein in the different subjects or the polymorphism of the size. Enzyme immunoassays can lead to size-based errors if antibodies directed against the epitopes of the variable portion of the Lp(a) particles are used. In addition, these tests involve the use of fixed size calibrators, which are often not representative of the heterogeneous dimensions of the LP (a). Therefore, diagnostic tests such as MS-based ones, allow a better evaluation of the concentration of Lp(a) both in case of low concentration of Lp(a) in individuals with large particles, and for high concentrations in patients with small particles.
The results of this study therefore support the use of the MS-based approach for assessing the levels of Apo (a) in serum.
As previously demonstrated by Lassman and colleagues, the LFLEPTQADIALLK (LFLE) peptide is unique to Apo (a) (Lassman 2014), as it is present only in the constant portion of Apo (a). Thus, after digestion with trypsin, the LFLE peptide signal area should be proportional to the molar concentration of Apo (a), independent of the heterogeneity of the molecular weight and its LFLE * labeled internal standard can be used for normalize matrix effects due to coelution of serum components. Lassmann and colleagues provided information regarding Apo (a) size analysis by HPLC MS/MS. GTYSTTVTGR (GTYS) peptide is included as an indicator of KIV2 copy quantity. The greater the size of Apo (a), the greater the number of KIV2 repeats, and the greater the concentration of the GTYS peptide, after digestion with trypsin, compared to that of the LFLE peptide.
To describe the average size of Apo (a) it could therefore be used the ratio between the areas of the GTYS peptide and of the GTYS * standard divided by the ratio between the areas of the LFLE and

LFLE * peptide. This method has limitations. In fact, if Apo (a) at the serum level is expressed as a single isoform, this MS-based method could replace the standard method for evaluating the dimensions of Apo (a) or SDS-PAGE electrophoresis.
Since the majority of people, however, express two different allelic forms, which lead to the encoding at least of two isoforms of Apo (a) with different dimensions, the value obtained by HPLC MS / MS must be considered as an average dimension that derives from 2 or more isoforms of Apo (a). Beyond this, HPLC MS/MS method constituted an excellent method in this study for the evaluation of the size distribution of serum apo (a) isoforms.
Consistent with previous studies, LDL and Lp (a) levels following apheresis were reduced (Adorni 2012).
MS-based method turned out to be a valid method for checking and confirming the results obtained with the traditional Ab-based method, also considering the low volume required for the analysis of only 4 μl.
In this study it was confirmed once again that CLC is a pro-atherogenic parameter, although the relationship with atherosclerosis at a clinical level has not yet been confirmed. Beyond this, the role of cholesterol in relation to CAD risk is recognized, as its accumulation in the macrophage cell leads to the induction of the formation of foam cells, which are the basis for the formation of atheroma (Moore 2018). Furthermore, as highlighted in this study, this atherogenic evolution may be related to the degrees and characteristics and to the dyslipidemic phenotype in vivo (Wong, Kyle 2011). Given the involvement of proatherogenic lipoproteins in the accumulation of cholesterol (Favari 2018; Rothblat 2002), we focused on the CLC of these patients with high levels of Lp (a) and CAD undergoing apheresis or not, also considering that this parameter is a directed way to investigate this.
The results of these patients suffering from high levels of Lp(a) and CAD allow to observe a direct association between the dimensions of Lp (a) and the CLC, in particular the greater the lipoprotein dimension, the lower the CLC. On the other hand, a relationship between CLC and the concentration of Lp (a) was not found.
This was one of the first studies on the non-dimensional polymorphism of the LPA gene in patients on apheretic therapy.
Through this study, two new variants, of still unknown clinical value, were identified. Given the limited number of samples in this study, it was not possible to individualize the role of each variant with respect to CV risk. Therefore, further studies are needed to establish the clinical significance of these variants.

Part II: HDL-Mediated Cholesterol Efflux and Plasma Loading Capacity in subject with metabolically or genetically driven NAFLD

Aim

NAFLD, a common hepatic disease in the Western world, shows as a salient feature the presence in the liver of an increase of TG over 5%, not caused by secondary causes of steatosis, such as alcohol abuse, autoimmune diseases, viral infections, etc (Younossi 2016). The risk factors of NAFLD, in addition to being the same as for CV diseases, are the key elements of the metabolic syndrome, which are dyslipidemia, insulin resistance, T2DM and obesity (Lonardo 2012; Younossi 2019).
Despite the role of metabolic changes in the development of the disease (Piccinin 2019), studies also demonstrate the existence of genetic factors that are able to induce the development of hepatic steatosis (Anstee and Day 2015); one of the most important genetic determinants of this disorder is recognized to be a variant of the protein belonging to the potato family, namely PNPLA3, which is associated with lipid droplets, where it tends to hyper-accumulate in the presence of the mutated form I148M (*rs738409*) (Romeo 2008). Although this form of steatosis arises as a liver disease, characterized by hepatic morbidity and mortality, the association with an increase in ASCVD risk is now known (Baratta 2020; Marjot 2020; Targher 2016). Despite the identification of various pro-atherogenic pathways (Lonardo 2016), it was not defined whether this accumulation of lipids in the liver was sufficient to determine the development of atherosclerosis starting from NAFLD. In a study conducted by Di Costanzo and colleagues was evaluated an index of subclinical atherosclerosis, i.e. the carotid intimal media thickness (C-IMT) in two groups of NAFLD patients, one on a genetic basis due to the *rs738409* variant of the previously mentioned protein, the other on a metabolic basis. In this analysis it was found that the C-IMT index was higher in patients with the metabolic disturbance, while in patients with the genetic variant the index was comparable to control subjects (Di Costanzo 2017). A confirmation of this data also came from Castaldo and co-workers who reported that the PNPLA3 *rs738409* form of NAFLD was not associated with variations of C-IMT (Castaldo 2020), and also through Mendelian randomization studies it was possible to observe how this genetic form of NAFLD was not associated with ischemic cardiomyopathy (IHD), but with an increased risk of liver-based morbidity (Lauridsen 2018).
The cause of this different type of risk between the two NAFLD variants is not clear, and this may be due in part to variations in some anti-atherogenic features.
An important class of lipoproteins that play a protective role against atherogenesis are HDL (Camont 2011), capable of promoting the first step of RCT, namely the cellular cholesterol efflux (Favari 2018; Khera 2011; Rohatgi 2014). Cellular cholesterol efflux is favored thanks to the interaction of the different classes of HDL with membrane transporters, such as ABCA1 and ABCG1. The first transporter, in particular, is able to transfer free cellular cholesterol to lipid-free Apo AI, known as preβ-HDL, while the second transporter is able to transfer cholesterol to mature HDLs, which are

also involved in other two mechanisms, namely passive diffusion (PD)-mediated efflux and SR-BI mediated efflux (Phillips 2014).

All these efflux mechanisms are opposed to the accumulation of cholesterol in macrophages, and so to the formation of atheromatous plaque (Phillips 2014). The efficiency of these HDL subclasses can be evaluated through the cholesterol efflux capacity (CEC).

The opposite mechanism capable of influencing the atherogenic process is the ability of serum to transfer cholesterol to macrophages, represented by the cholesterol loading capacity (CLC) (Adorni 2012; Zimetti 2015).

There is little evidence of serum CEC from NAFLD patients, and no information is present regarding serum CLC; in addition in the two forms of NAFLD, described above, i.e. the metabolically determined and the genetically determined, neither the CEC nor the CLC are known.

Therefore, the aim of the first project of this thesis was to examine CEC and CLC in patients with NAFLD on a metabolic basis and in patients with genetic NAFDL in comparison with healthy subjects. This project was done in collaboration with Sapienza University of Rome.

Materials and Methods

Study partecipants

For the study were enrolled 39 subjects, distinguished in 29 patients with NAFLD and 10 subjects without NAFLD. Control subjects are wild type for PNPLA3 gene and are referred to as controls. The patients were divided into two groups: the first group consisting of NAFLD patients with metabolic disorders with PNPLA3 (I148I) gene in wild type form, indicated as group M (n = 19, metabolic NAFLD); the second consists of patients carrying the homozygous PNPLA3 rs738409 variant (M148M), without metabolic disorders, indicated as group G (n = 10, genetic NAFLD).
The patient sera of group M come from the Department of Internal Medicine and Medical Specialties of Sapienza University of Rome, while the control subjects and patients of group G come from the Department of Immunohematology and Transfusion Medicine Sapienza University of Rome.
The anthropometric and metabolic characteristics of the study subjects are shown in Table II.
Thanks to nuclear magnetic resonance with spectroscopy, the existence of hepatic steatosis has been identified, while the excess consumption of alcohol is detected with the help of the AUDIT questionnaire, and alcohol abuse was excluded as a secondary cause of NAFLD if less than 30 g/day in men and 20 g/day for women (Sanyal 2011).
BMI above 30 kg/m^2 was considered as the threshold above which the patient was considered obese. For the quantification of insulin resistance (HOMA-IR) we resorted to the use of the homeostasis model assessment, HOMA (Matthews 1985), while the diagnosis of T2DM was carried out according to WHO criteria (American Diabetes Association 2010). The diagnosis of metabolic syndrome (MetS) was made following the criteria of the NCEP-ATP III Expert Panel of the US National Cholesterol Panel (Grundy 2005).
Patients enrolled in the study were not taking lipid-lowering therapies.

Cell lines

The cell cultures used were kept in sterile conditions as mentioned before and manipulated using a vertical laminar flow hood. Cell cultures are kept in an incubator at 37 °C, at 5% CO2 and with a humidity of 90-95%.
For the morphological observation of the cells was used a Leica Microsystems inverted optics microscope.
Cells were kept in line under the following conditions:
• The J774 mouse macrophage cell line was grown in DMEM (Euroclone) added before use with 1% penicillin/streptomycin (P/S, Gibco) and 1% geneticin (Gibco) and supplemented with 10% of fetal bovine serum (FBS, Euroclone). To be used in experiments, the cell line must reach approximately

100% confluence. For the detachment of the monolayer, after washing in Phosphate Buffered Saline (PBS, Euroclone), cell scraper is used with which a mechanical detachment occurs.

• CHO-K1 is a non-transfected cell line, from which CHO-L3 are derived. They are maintained with HAM's F-12 (Euroclone) added with 1% of L-glutamine (Sigma-Aldrich), 1% of P/S and 10% of FBS. For the subcultivation and seeding steps, the cells are enzymatically detached through the use of trypsin (Life Technologies).

• CHO-L3 is a cell line obtained by transfection of the CHO-K1 line with a plasmid containing the ABCG1 gene. They are kept in HAM's F-12 with 1% L-glutamine, 1% P/S, 0.2% zeocin (Life Technologies) and with 10% FBS. The same mechanism used for CHO-K1 is used for the subcultivation and seeding steps of CHO-L3.

• THP-I is a line of human monocytes, which grow in suspension in RPMI 1640 containing HEPES, Sodium Pyruvate 1mM and glucose 2.5mg/ml, gentamicin 50 mg/ml, ß-mercaptoethanol 50 mM, enriched at the time of use with 10% FBS. For the subcultivation this cell line is maintained in RPMI 1640 at 10% FCS. Otherwise, for seeding, THP-I are differentiated to macrophages by treatment with PMA at a concentration of 100 ng/mL.

• Fu5AH is a line of rat hepatomas grown in DMEM (Euroclone) added with 0.5% gentamicin (Thermo Fisher Scientific) and 10% FBS at the time of use. For the subcultivation and sowing steps, the Fu5AH were detached with the enzymatic method through the use of trypsin.

Evaluation of Passive Diffusion (PD), Total and ABCA1-mediated CEC

Total-, Passive diffusion (PD)- and ABCA1-mediated efflux are evaluated with J774 cell line by using a standardized radioisotope method (Favari 2018; Khera 2011; Rohatgi 2014; Zimetti 2017). Briefly, the cells are seeded in 24-well plates (Corning) at the density of 250000 per well. 24 hours after seeding, the cell monolayers are radiolabelled with [1,2-3H]-cholesterol 2μCi/mL (Perkin Elmer) in DMEM added with 1% FCS. 24 hours after radiolabelling, cells are treated with DMEM at 0.2% bovine serum albumin (BSA, Sigma-Aldrich), in presence of 0.3 mM cpt-cAMP (Sigma-Aldrich) capable of inducing ABCA1 transporter, and used to evaluate total efflux given by the contribution of PD and ABCA1, or in absence to evaluate PD.

In the labeling and equilibration phase ACAT inhibitor is added at a concentration of 2 μg/ml (Sandoz 58035, Sigma-Aldrich).

After 18 hours, the efflux promotion phase is carried out with patient sera at 2% (v/v) for 4 hours. By using the scintillation liquid, Opti-fluor (Perkin-Elmer), the radioactivity (cpm) in the culture medium is evaluated; in particular cellular cholesterol efflux can be evaluated thanks to a set of cells stopped

at time 0 (t0), before adding the test sera. The formula for evaluating the percentage of cellular cholesterol efflux is as follows:

$$\% \text{ efflux} = (\text{cmp medium} / \text{cpm T0}) \times 100$$

To analyze the cellular [^{3}H]-cholesterol content in the cell monolayers, lipid extraction is carried out by adding 0.6 mL of 2-propanol (VWR).

In the experiments, a pool of normolipidemic human sera is used as an internal standard. The efflux values of this standard were used for the normalization of the experiments, made to eliminate the inter-essay variability (Zanotti 2012).

The ABCA1-mediated cholesterol efflux is calculated by subtracting the percentage of efflux of non-stimulated cells from the percentage of efflux of cells stimulated with cAMP.

Measurement of ABCG1-mediated CEC

CHO-L3 cells, obtained starting from CHO-K1, following transfection with a plasmid containing human ABCG1 transporter, are used for the evaluation of cholesterol efflux mediated by this transporter.

After 24 hours from the seeding procedure, which takes place at the density of 25000 cells per well in 24-well plates, the cells are treated with [1,2-3H]-cholesterol 1µCi/mL in a medium containing 5% FCS. After 24 hours from labeling phase, the equilibration phase is carried out with medium containing 0.2% BSA for 90 minutes, at the end of which the cells are incubated with a medium containing 1 % (v/v) patient's serum for 6 hours. The efflux of cholesterol is measured as the ratio between the radioactivity released by cells exposed to sera in the culture medium and the cells at t0.

Also in this case, a pool of normolipidemic human sera is used as an internal standard, and for the normalization of the experiments. The ABCG1 mediated efflux percentage is calculated by subtracting the efflux value of the cells not expressing the transporter from the efflux value of the transfected cells.

Measurement of SR-BI-mediated CEC

Fu5AH are plated at a density of 100000 cells/well in 48-well plates for 24 hours in 10% FBS medium. After 24 hours, the cells are labeled with [1,2-3H]-cholesterol 2µCi/mL (Perkin Elmer) in the presence of 1% FBS and ACAT inhibitor at a concentration of 2 µg/mL. Subsequently, the equilibration phase takes place with 0.2% BSA in the presence of an ACAT inhibitor. Two hours before the efflux phase, a pre-treatment lasting 2 hours takes place in the presence of SR-BI inhibitor

(Block Lipid Transfer-1 10 μM, ChemBridge) in order to evaluate the PD process, or in its absence to evaluate SR-BI-mediated efflux and PD-mediated efflux. Subsequently, the efflux phase is carried out lasting 4 hours in presence of 2% (v/v) of the subjects' sera under examination.

The efflux of cholesterol is measured as the ratio between the radioactivity released in the culture medium and the cells at t0.

Once again, a pool of normolipidemic human sera (internal standard) is necessary for the normalization of the experiments. SR-BI mediated efflux percentage is calculated by subtracting the value of cells treated with SR-BI inhibitor from the efflux value of cells not treated with it.

Evaluation of Serum-Mediated Macrophage CLC

For this parameter THP-1s are used, which are sown in 24-well plates with a density of 500000 cells per well, in a medium containing 10% of FBS and PMA at a concentration of 100 ng/mL. After 72 hours from seeding, during which takes place the differentiation to macrophages, the cells are incubated for 8 hours with 5% (v/v) of patients' serum, who act as cholesterol donors.

At the end of this period, after washing with PBS, macrophages are lysed in a solution of 1% cholic acid and 50 U/mL DNA-asi and left to stir overnight. CLC is evaluated as μg of cholesterol per mg of protein.

A fluorimetric kit, Amplex Red Cholesterol Assay Kit (Molecular Probes by Life Technologies) is used to quantify the cholesterol content in the lysates, while the bicinconinic acid method is used to evaluate the protein content in the lysates.

Statistical analysis

GraphPad 9.0 software was used to perform statistical analyzes.

For variables with normal distribution the data are reported as mean and standard deviation, while for data not normally distributed the data are reported as median (25-75th percentile). ANOVA or t-Student test were used to analyze the differences between groups in case of parameter variables, while Kruskal-Wallis and Mann-Whitney test for non-parameter variables. Spearman's correlation was used to evaluate correlations. $P \leq 0.05$ are considered statistically significant.

Results

Anthropometric and metabolic characteristic of study groups

Table II summarizes the anthropometric and metabolic characteristics of the grouped patients. The subjects were selected on the basis of age and gender, for this reason they do not differ for these characteristics, on the contrary they are significantly different for BMI, waist circumference (WC), systolic and diastolic BP, HDL-C, total TG, Apo A-I, fasting insulin and glucose levels, as well as HOMA-IR, and liver enzymes (all $p \leq 0.03$). MetS is present in 42.1% of subjects with metabolic NAFLD, while TD2M is present in 15.8% of patients with metabolic NAFLD. As for pharmaceutical treatments, 15.8% of patients use metformin and repaglinide, which are hypoglycemic drugs, and 61.1% use antihypertensive drugs.
The levels of adiponectin (ADP) are significantly lower in patients belonging to group M ($p < 0.001$) compared to both controls and to patients belonging to group M, this finding is an additional confirmation of metabolic disorders in group M.
As regards group G, a significant reduction in HDL-C ($p = 0.031$) and Apo A-I ($p = 0.009$) has been observed compared to control subjects.
The amount of hepatic fat (HFF%) increased compared to control subjects both in patients of group M and in patients of group G. Another interesting fact is that after the division of group M based on the presence of MetS, it was possible observe how NAFLD patients, who presented MetS, compared to patients with NAFLD but without MetS, had a higher age ($p = 0.026$), BMI ($P = 0.008$), WC ($p = 0.021$), levels of fasting glucose (0.012), fasting insulin ($p = 0.012$), and insulin resistance ($p = 0.008$) (data not shown in the table).

		NAFLD		
	Controls	GroupG	GroupM	p Value
N	10	10	19	
Age, Years	55,9 ± 6,9	54 ± 8,5	55,6 ± 3,8	0,7
Males, %	80	78,9	80	0,9
MetS, %	0	0*	42,1	0,005
BMI, kg/m2	25,5 ± 2,9	27,2 ± 3,6	30,8 ± 5,8 ***	0,01
WC, cm	93,9 ± 9,4	99,0 ± 12,4	107,5 ± 10,1 ***	0,006
Smokers, %	50	40	15,8	0,12
T2DM, %	0	0	15,8	0,18
HFF, %	2,06 ± 1,61 **	36,1 ± 22,4	32,0 ± 19,6 ***	<0.001
Systolic BP, mmHg	115 (103,7-120)	120(112,5-130)*	130 (120-140)***	0,003
Diastolic BP, mmHg	70 (65-80)	80 (72,5-84,2)	80 (75-85)***	0,015
Total cholesterol, mg/dL	206,8 ± 28,5	206,4 ± 35,2	208,2 ±27,8	0,9
HDL cholesterol, mg/dL	64,2 ± 10,3 **	52,6 ±11,8	45,6 ±12,9	0,002
LDL cholesterol, mg/dL	125,1 ± 30,2	133,1 ±27,9	126,9 ±24,5	0,7
Total triglycerides, mg/dL	85.5 (59.5–102.7)	88 (84.7–106) *	159 (125–210) ***	0.005
ApoB, mg/dL	97.9 ± 16.5	97.5 ± 16.5	92.0 ± 21.3	0,6
ApoAI, mg/dL	133 ± 14.4 **	113.9 ± 14.6	106.6 ± 20.0 ***	0,002
Fasting glucose, mg/dL	78.5 ± 10.0	82.9 ± 13.8	98.0 ± 21.2 ***	0,1
Fasting insulin, U/L	5.3 (4.2–7.9)	5.9 (4.9–10.9) *	12.7 (8.6–18.9) ***	0,1
HOMA -IR	0.9 (0.9–1.6)	1.2 (0.9–2.2) *	2.9 (1.8–4.2) ***	<0.001
ADP, ng/dl	6596.8 ± 3179.6	4791.5 ± 1796.4 *	901.0 ± 413.8 ***	<0.001
AST, U/L	29.5 (19.7–36.5)	31.5 (22.2–33.2) *	20 (18–26)	0,026
ALT, U/L	13 (10–22)	16 (13.7–19.5) *	34 (21.5–42.5) ***	<0.001
y-GT, U/L	18.5 (13.2–24.5)	20.5 (15.2–29.2)	25.5 (20.7–38.2) ***	0,05

Table II: Anthropometric and metabolic characteristic of groups in study. Non-normal distributed variables are presented as median (inter quartile range), while for normal distributed variables the data are presented as median ± SD. The comparison between group M and group G is indicated with * p <0.05, while the comparison between group G and the controls is indicated with ** p <0.05, and the comparison between group M and controls with *** p <0.05. ADP=adiponectin; ApoAI= apolipoproteinA-I; BMI= body mass index; HDL-C=high-density lipoprotein cholesterol; AST=aspartate aminotransferase; ALT=alanine aminotransferase; HFF=hepatic fat fraction; HOMA-IR=homeostasis model assessment of insulin resistance; BP=blood pressure; MetS=Metabolic syndrome; T2DM=Type 2 diabetes; WC=waist circumference.

Comparison between Groups of HDL-Mediated CEC

Figure IIa shows total CEC assessed in J774 macrophages, following stimulus with cAMP. Total CEC of group M is significantly lower than group G (17.1 ± 1.1% vs 21.0 ± 0.9% p <0.001) and the control group (17.1 ± 1.1% vs 21.1 ± 1.0% p <0.001). Thus, no difference was observed in total CEC between control patients and group G, while group M showed a reduced total CEC of 18.6% compared to group G, while a 19% reduction was observed in comparisons with the controls (p <0.001). In panel IIb is indicated total CEC following the subdivision of patients according to the

presence or absence of MetS. It can be seen a progressive decline from group G to group M MetS- and to group M/MetS+ (p <0.001).

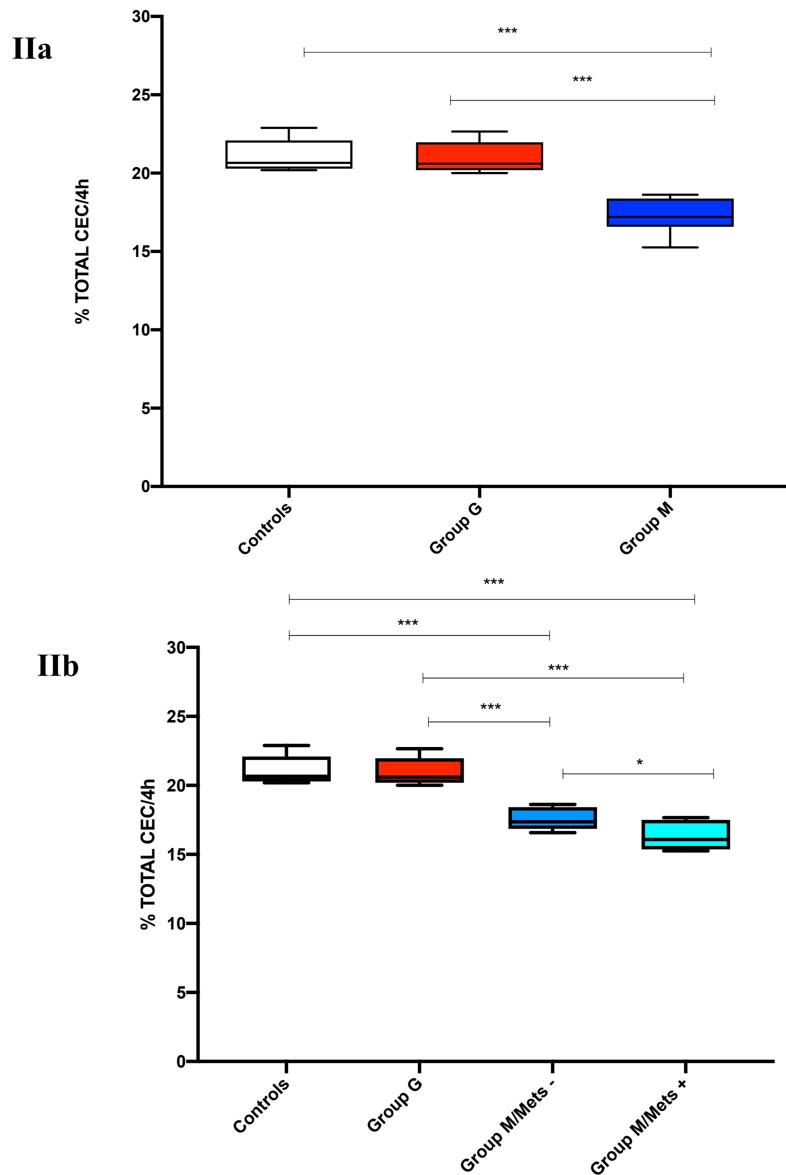


Figure IIa-b: Total HDL-CEC depending on NAFLD classification is shown in Panel A, while in Panel B is shown depending on presence of MetS diagnosis. In the panel A, through a univariate analysis, it was observed that group M has a lower total efflux independent of Apo A-I levels and HFF% (Padj <0.001). In panel B, through a univariate analysis, it was observed that patients in the M/MetS+ group have a lower total CEC regardless of age, HFF% and Apo A-I levels in comparison with M/MetS- group (Padj = 0.010). Cholesterol efflux data are represented as median and IQR. As regards the significance *** for p <0.0001; * for p = 0.029. Group M indicates metabolic NAFLD group; Group G indicates genetic NAFLD group; Group M/MetS- indicates metabolic NAFLD group without MetS diagnosis; Group M/Mets+ indicates metabolic NAFLD group with MetS diagnosis.

Moving on to the different efflux pathways shown in figure IIc, the patients of group M showed a significant reduction in CEC mediated by ABCG1, ABCA1 and PD compared to the patients of group G and to the control group (p <0.001); on the contrary it was not seen an SR-BI-mediated CEC

reduction. Figure IId shows CEC following the subdivision of patients in group M according to the presence or absence of MetS. A progressive decline in CEC mediated by ABCG1 can be observed from control group to G group, and from M/MetS- group to M/MetS+ group (p for trend = 0.031). In addition, no differences were observed with respect to these groups for CEC mediated by the ABCA1, SR-BI and PD.

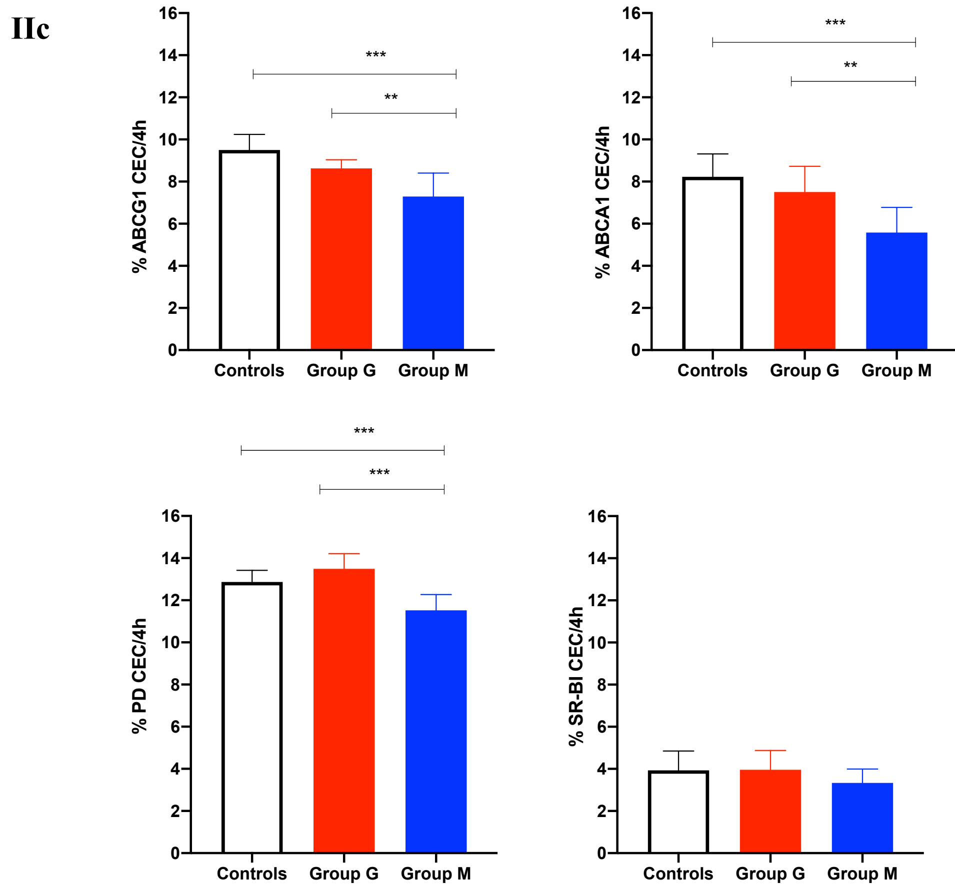


Figure IIc: In Panel IIc is reported CEC mediated by ABCG1, ABCA1, PD and SR-BI according to NAFLD classification groups. CEC is expressed as mean and SD. For the significance *** for p < 0.0001; ** for p >= 0.002.

IId

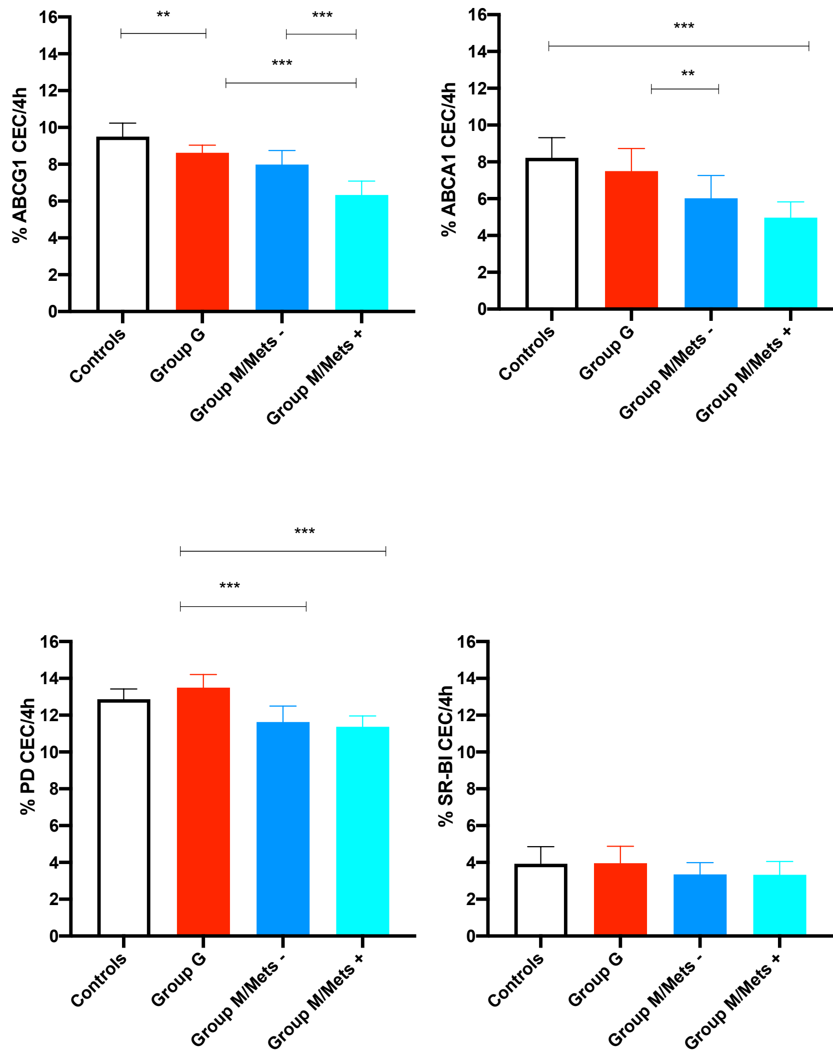


Figure IId: In Panel IId is reported CEC mediated by ABCG1, ABCA1, PD and SR-BI according to the presence or absence of MetS. CEC is expressed as mean and SD. For the significance *** for $p < 0.0001$; ** for $p >= 0.002$.

Furthermore, given that different HDL-C levels was found between group M and group G, as reported in Table II, we decided to compare CEC in function of the quartiles of the different HDL-C levels (Figure IIe). It was observed that patients of group M showed a lower total CEC than group G for equal levels of HDL (all $p <0.05$), while no differences were observed between patients of group G and controls.

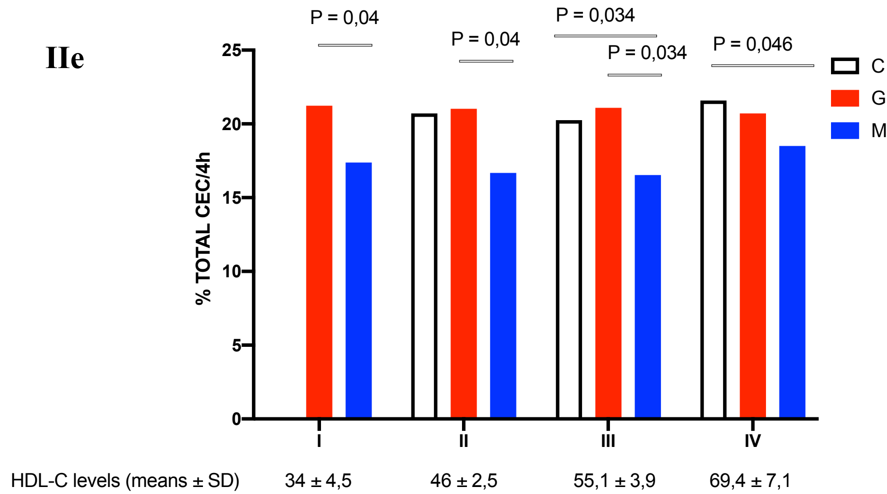


Figure IIe: The figure shows total CEC of patients divided into groups according to the quartiles of HDL-C. Through the Mann Whitney test, significant differences were found in all groups of quartiles.

To further investigate the mechanisms responsible for these variations in CEC between group G and group M, we decided to evaluate the variation of preβ-HDL as well as the ratio between non-esterified cholesterol and total cholesterol (UC/TC) between different groups, represented respectively in figure IIf and IIg. As shown in figure IIf, patients of group M have a lower concentration of preβ-HDL than group G, despite there is no statistical significance (12.9 ± 5.9% vs 16.2 ± 3.8%). Figure IIg shows the UC/TC ratio which is significantly higher in patients of group M than in controls group and group G, which remains even after adjustment for ApoAI and HFF% (Padj = 0.015).

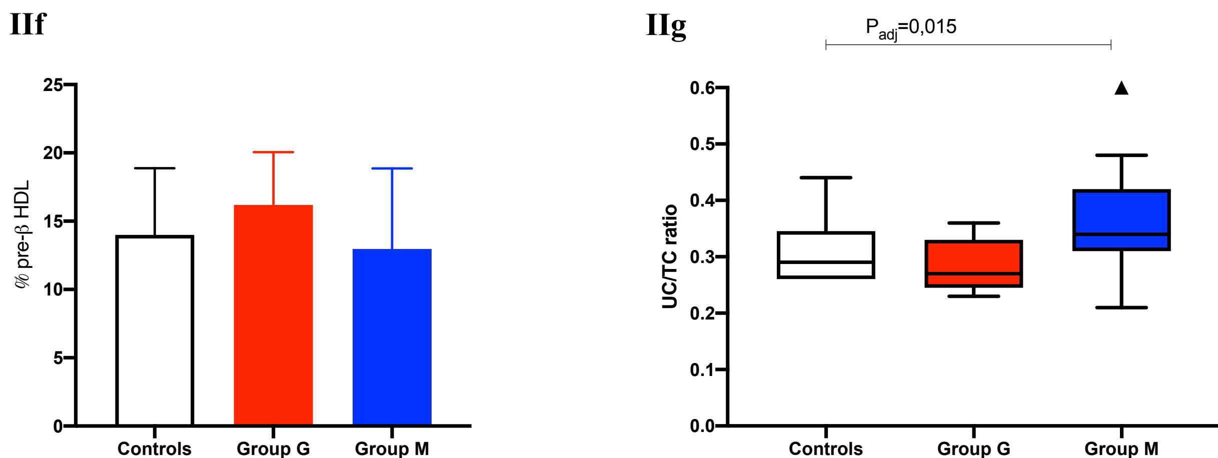


Figure IIf-IIg: in figure IIf is represented the concentration of pre-β HDL in the three groups under study, represented as mean ± SE, while in figure IIg the UC / TC ratio, indicated as median and IQR, is represented by box plot. Following the adjustment for Apo A-I and HFF% Padj = 0.015.

Comparison between Groups of Plasma-Mediated Macrophage CLC

Both the efflux of cholesterol and the entry of cholesterol into the cell determine the overall cholesterol content within the cell (Adorni 2012). For this reason it was tested the ability of the patients' serum to load cholesterol in macrophages (CLC). In Figure IIh it can be seen that patients of group M have a higher CLC than patients of group G or controls (p <0.001). Figure IIi shows CLC of patients following the subdivision according to the presence or absence of MetS. Patients in group G have reduced CLC (27.5 ± 1.5 µg/mg) compared to group M/MetS– (33.5 ± 1.6 µg/mg, p <0.001) and to M/MetS + group (35.9 ± 1.8 µg/mg, p = 0.003).

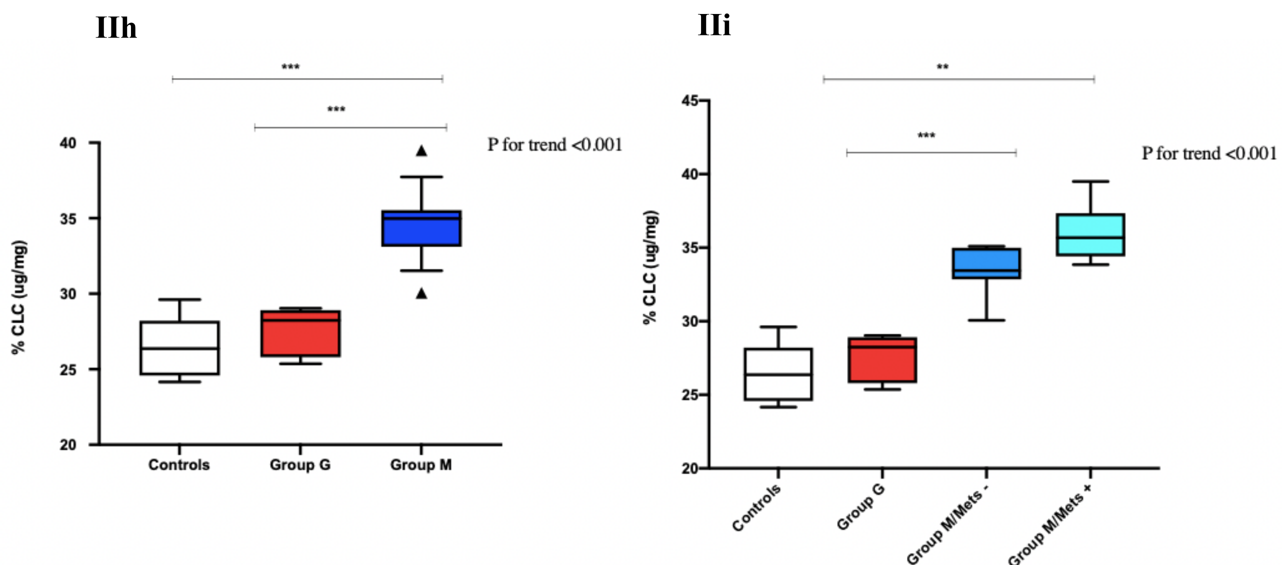


Figure IIh-IIi: comparison of the CLC of different groups. In figure IIh CLC is represented according to the classification of NAFLD (*** p <0.0001), while in figure IIi CLC of patients is represented after subdivision based on the presence or absence of MetS (*** p <0.0001 , ** p = 0.003). Data are represented as median and IQR of the cholesterol content within the cell following its exposure to the serum of the patients under examination.

Relationship of CEC and CLC with Clinical Parameters

As regards the correlations between total CEC and CLC with anthropometric and biochemical parameters, evaluated in the whole population, and therefore not divided into the different groups, it is worth mentioning the negative correlation between CEC and obesity indices (r = - 0, 61, p <0.001), as well as with HOMA-IR (r = -0.72, p <0.001), systolic and diastolic BP (respectively r = -0.41, p = 0.011 and r = -0.34, p = 0.042), total triglycerides (r = 0.52, p = 0.001), ALT levels (r = 0.57, p <0.001) and HFF% (r = -0.44, p = 0.005). Furthermore, CLC is positively correlated with BMI (r = 0.43, p = 0.007), HOMA-IR (r = 0.56, p <0.001), systolic and diastolic BP (r = 0.59, p <0.001 and r = 0.47, p = 0.004, respectively), total triglycerides (r = 0.59, p <0.001), ALT levels (r = 0.55, p <0.001) and HFF% (r = 0.52, p = 0.001) (data not presented).

It is also worth mentioning the linear association existing between the concentrations of adiponectin with total CEC (β = 0.70, p <0.001) and with CLC (β = -0.77, p<0.001), which even following the **IIl** ents for age, sex, smoking, BMI, HDL- **IIm**)MA-IR remains significant (all Padj <0.003) (Figure IIl-IIm). Furthermore, an inverse correlation was also highlighted between CEC and CLC, again in the population not subdivided into the group (β = -0.83, p <0.001) (data not presented).

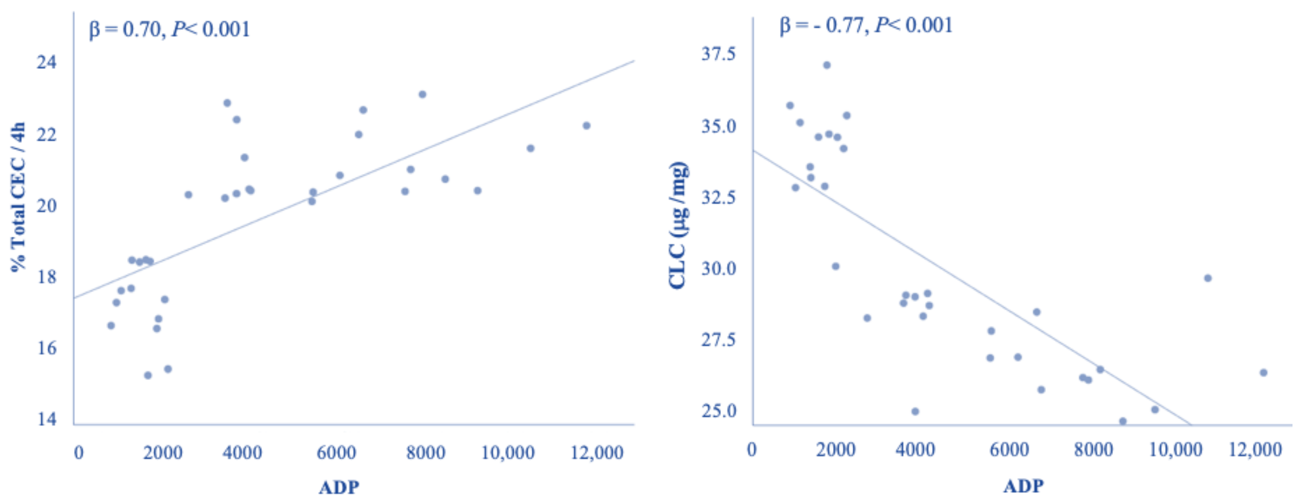


Figure IIl-IIm: Linear regression analysis between CEC, CLC and adiponectin levels. In figure IIl is reported the linear regression analysis between CEC and adiponectin levels. R-squared is 0,49. In figure IIm is represented the linear regression analysis between CLC and adiponectin levels. R-square is 0,60.

Discussion

What is evident from this study is that patients with metabolic NAFLD present changes in HDL-dependent CEC and also in CLC compared with control subjects and patients with genetic form of NAFLD. In addition, these alterations are even more marked in NAFLD patients in presence of MetS. Therefore, in patients suffering from metabolic NAFLD there is an imbalance in cellular cholesterol homeostasis, which leads to pro-atherogenic phenomena, due on one hand to the inability of HDL to promote the efflux of cholesterol from cell and on the other hand due to the greater ability to load cholesterol into cells, as evidenced by the use of human macrophages.
Already exist studies that analyze the relationship between HDL-CEC and hepatic steatosis (van den Berg 2018; Fadaei 2018); Fadaei and co-workers showed that in patients with metabolic NAFLD there is a decrease in total CEC compared to control subjects, largely due to a decrease in efflux mediated by ABCA1 transporter, which remained significant even after adjustments for confounding factors, as opposed to total CEC which lost significance after adjustments for risk factors (Fadaei 2018). Van den Berg and colleagues also demonstrated a reduction in total CEC, independent of HDL levels, and an increase in chronic low-grade inflammation in NAFLD patients (van den Berg 2018).
Through this second project it was therefore possible to broaden the knowledge on the relationship between NAFLD and cholesterol efflux mediated by other pathways, such as those mediated by the transporters ABCG1, SR-BI and that mediated by passive diffusion. MRI/MRS was used to quantify fat content in the liver. It was found that CEC was not reduced in NAFLD patients with the genetic variant of PNPLA3, who had a liver fat content comparable to that of patients with metabolic NAFLD, confirming that excess fat in the liver is not a sufficient condition to compromise the functionality of HDL. A variation in the composition of HDLs may be responsible for the lower efficiency of these lipoproteins in promoting the efflux of cellular cholesterol in subjects with metabolic NAFLD. Thanks to the subdivision of the total CEC according to the quartiles of HDL-C, it was possible to exclude an involvement of the HDL content in the reduction of the ability of these particles to favor the efflux of cholesterol. Therefore, the alternative hypothesis concerns the possible alteration of the qualitative composition of the HDL subclasses. Indeed, one evidence in favor of this is the reduction of ABCA1-mediated efflux, a mechanism due to preβ-HDL (Favari 2009). Furthermore, it was established that patients in group M have a lower amount of this HDL subclass than patients in group G.
In addition, a decrease in LCAT activity was observed in patients with metabolic NAFLD, which led to an increase in non-esterified cholesterol levels, because this enzyme esterify preβ-HDL cholesterol leading to the formation of mature HDL (Favari 2009; Phillips 2014).
This last result is consistent with the positive correlation between CEC and LCAT activity highlighted by Fadaei and colleagues (Fadaei 2018) and could also be the cause responsible for the decrease in

CEC mediated by ABCG1 and PD, which are mechanisms that foresee the preferential acceptor of cholesterol mature HDL (Phillips 2014).

Furthermore, the decrease in SR-BI-mediated CEC in patients of group M is due to the fact that this flow path depends on the gradient concentration of cell membrane cholesterol and cholesterol acceptors (Adorni 2012). In this study the decrease in cholesterol efflux, experienced by patients with metabolic NAFLD, may be caused by the reduction of preβ-HDL as well as by the lower LCAT activity, which results into an increase of non-esterified cholesterol and into a reduction of the formation of mature HDL. However, in this project it was not possible to directly measure LCAT activity.

Patients affected by NAFLD and MetS have a total CEC reduced by 7.4% compared to patients in group M without metabolic syndrome and by 22% compared to patients in group G, thus demonstrating the additional decrease in HDL function, which happens in case of diagnosed presence of MetS. Some studies are in favor of reduced HDL function, expressed as the ability to promote cholesterol release, in the presence of metabolic disturbances (Annema 2016; Borggreve 2003; Gall 2021). Gall and colleagues reported a correlation between CEC and MetS, with a decrease in CEC from 4 to 11% in presence of increasing number of diagnostic criteria for MetS (Gall 2021).

It is known that the composition of the phospholipids present in HDL influences CEC (Kobayashi 2006), therefore variations in the composition of these lipids could be responsible for this different functionality. Further evidence in favor of this finding comes from a study conducted by Meikle and colleagues that demonstrates a negative association between phosphatidylcholine and sphingomyelin, present in HDL, and insulin resistance, present in T2DM or in the pre-diabetic status (Meikle 2013). These data support the hypothesis that an additional decrease in HDL functionality, expressed as CEC, in patients with NAFLD and MetS may be caused by alterations in phospholipids. In addition, it is known that ABCG1-mediated efflux depends on the phospholipid gradient (Sano 2007), therefore alterations in phospholipids may be responsible for the decrease in ABCG1-mediated efflux observed in this study.

Furthermore, given the fact that the amount of cholesterol in the cell depends not only on the efflux but also on the influx, we decided to evaluate cholesterol loading capacity (CLC) of patients' serum, which has not been evaluated in this disorder yet.

Through this analysis it was possible to observe a significant increase in CLC in patients of group M compared to those of group G.

Since CLC is predominantly LDL dependent, the composition and physicochemical properties of this lipoprotein could influence the parameter (Adorni 2012; Ronda 2015), although no correlation was

observed between LDL levels and CLC. Therefore, the mechanisms responsible for this result is still to be understood.

Given the results obtained in this study, it is possible that the alteration of these two parameters, namely CEC and CLC, observed in patients with metabolic NAFLD, but not in the genetic one, may be responsible, at least in part, for the link between metabolic steatosis and atherogenesis, as well as the different CV risk observed in the two types of NAFLD.

However, the relationship between HDL functionality and C-IMT could not be determined due to the small number of samples. Fadaei and colleagues demonstrated an association between ABCA1 mediated-CEC alteration and subclinical atherosclerosis in this disease (Fadaei 2018); in fact, in this second project we found a relationship between adiponectin, endowed with anti-inflammatory and anti-atherogenic activities (Ebrahimi-Mamaeghani 2015), and CEC and also between adiponectin and CLC, which is in line with the latter data.

The results obtained from this study provide further information on a clinical level, because it allows to understand how metabolic NAFLD should be considered as a separate category. In fact, experts have recently introduced a new term, MAFLD, "fatty liver disease associated with metabolic dysfunction", to indicate this type of NAFLD characterized by metabolic disorders (Eslam 2020). The last fact to underline is that, as evidenced by these results, it is important to characterize the type of NAFLD in order to identify its CV risk as well.

Part III: Effects of Mediterranean vs lacto-ovo vegetarian diets on HDL function: the CARDIVEG study

Aim

CVD represent the major cause of death globally, with almost 18.6 million of deaths all over the world in 2019, and with an increase of 17.1% in the last decade (Arnett 2019). It is known that CVD risk is affected by different factors, such as lifestyle, including poor diet, tobacco smoking and physical inactivity. For this reason, healthy dietary patterns such as Mediterranean (MD) or vegetarian (VD) diets have been largely studied so far for their potential protective role on CVD (Dinu 2020).

These diets emphasize the consumption of plant-based foods, whole grains and legumes, while limiting refined grains, added sugars, processed foods and providing limited or no intake of poultry and meat, as in the case of VD.

These dietary patterns protect against atherosclerosis, being able to preserve lipids from oxidative stress, and possessing anti-inflammatory effects, as well as the ability to modulate the intestinal microbiota and the metabolites produced in the intestine, related to CVD (Dinu 2017; Pagliai 2020). An improvement in the lipid profile associated with HDL has been observed in diets rich in plant-based foods (Solá 2011).

HDLs represent a heterogeneous family of lipoprotein particles, which possess cardioprotective effects, and differs in terms of density, size, surface charge, protein composition and functions. The concentration of the different HDL subclasses would be inversely correlated with CV risk (He 2018), a data recently confirmed in a meta-analysis study in patients on statin therapy (Sampson 2012).

HDL, important in the physiological process of RCT, as well as in the inflammatory, oxidative, apoptotic and thrombotic pathways, have an impact on atherosclerosis (Kontush 2014). The first step of RCT is the cellular efflux of cholesterol, mediated by various mechanisms (Favari 2015). Although the therapeutic method for CVD reduction was essentially based on the increase in HDL levels, studies aimed at achieving this goal, using CETP inhibitors, have not shown any benefit in terms of CVD risk reduction (Armitage 2019; Tall and Rader 2018). Similar results were also obtained in randomized trials with niacin (Garg 2017; Mani and Rohatgi 2015). Interest has recently been placed on the cholesterol efflux mediated by HDL via ABCA1 (Rosenson 2012). In vivo studies have shown the existence of an inverse relationship between the specific ability of HDL to promote the cholesterol efflux from macrophages and coronary events, an association confirmed even after adjustments for HDL-C, Apo-AI and HDL particle number (Hunjadi 2020; Riggs and Rohatgi 2019).

There are various foods and nutrients capable of positively influencing the different functions possessed by HDL (Hernaez 2014; McEneny 2013); Interesting are the data from the PREDIMED study that demonstrate how a MD diet rich in extra virgin olive oil can improve the atheroprotective functions of HDL (Hernáez 2017). Today, however, there is no dietary or pharmacological strategy capable of increasing the CEC.

The aim of this third project, done in collaboration with Professor Francesco Sofi, University of Florence, was to evaluate the effect of two dietary types, namely MD and VD, on HDL functionality assessed as cholesterol efflux capacity, in clinically healthy subjects, through the use of data obtained from the CARDIVEG study, which was a randomized controlled dietary intervention study.

Materials and Methods

Study partecipants and design

This study involved 30 clinically healthy subjects (21 females, mean age was 51.29 ± 9.74 years), previously enrolled in the CARDIVEG study, a randomized, open, crossover study type that compared the effects of two types of diets, MD and VD, on various CVD risk factors (Sofi 2018). Details of the study were previously described elsewhere (Sofi 2018). Subjects were aged 18 to 75 years, with low to moderate CV risk (less than 5% at 10 years, according to the European Society of Cardiology). The study participants were recruited from the Clinical Nutrition Unit of the Careggi University Hospital, Florence, Italy.

Regarding the eligibility criteria included BMI greater than 25 kg/m^2 and the concomitant presence of at least one of the CVD risk factors: total cholesterol levels > 190 mg/dL; LDL cholesterol levels >115mg/dL; triglyceride levels >150 mg/dl; glucose levels >110 mg/dL but <126mg/dL. We excluded people with serious illness or unstable conditions, women who were pregnant or breastfeeding, people who took medication for any reason, who excluded meat, poultry or fish from their diet in the past 6 months, or who participated in a treatment program for the weight loss in the past 6 months.

After a 2-week run-in period, the participants were randomly assigned to MD or VD for 3 months and then moved on to the other treatment. Participants were instructed not to vary their lifestyle or physical activity habits and were not given any weight loss goals. Primary outcomes were changes in total body weight, body mass index, and fat mass from baseline, while secondary outcomes were changes in circulating CVD risk markers from baseline.

Dietary interventions and compliance

Both diets were low-calorie, but isocaloric to each other. The diets were characterized by 50-55% carbohydrate energy, 25-30% total fat and 15-20% protein.

As for MD, the meal plan included all food categories, including meat, meat-based foods, poultry and fish. The VD, on the other hand, did not include meat from any type of animal, neither its derivatives, nor fish or seafood, but it included eggs and dairy products.

For the evaluation of adherence to the MD was used the adherence score to the MD (Sofi 2017), and those participants who have at least a score of 10 on a scale ranging from 0 to 18 points were considered as adherents to it. Compliance with VD was analyzed using the modified version of the National Health and Nutrition Examination Survey (NHANES) Food Questionnaire and through a 24-hour dietary recall interview. Adherence to VD was defined as the absence of consumption of meat, meat products, poultry, fish and seafood in the diet of the participants.

Cell line

The cell culture used was kept in sterile conditions and manipulated with a vertical laminar flow hood. The cell line was kept in an incubator at 37 °C, at 5% CO2 and with a humidity of 90-95%. For the morphological observation of the cells was used a Leica Microsystems inverted optics microscope.

J774 mouse macrophage cell line was grown in DMEM (Euroclone) added before use with 1% penicillin/streptomycin (P/S, Gibco) and 1% geneticin (Gibco) and supplemented with 10% of fetal bovine serum (FBS, Euroclone).

Cell line must reach approximately 100% confluence to be used. For the detachment of the monolayer, after washing in PBS (Euroclone), cell scraper was used for a mechanical detachment.

Evaluation of HDL-CEC

Serum CEC mediated by ABCA1 cholesterol transporter and by the passive diffusion process were quantified. A highly standardized radioisotope technique was used to evaluate the ability of serum to promote cholesterol efflux, using a cellular model capable of overexpressing ABCA1 (Favari 2018; Khera 2011; Rohatgi 2014; Zimetti 2017). In particular, murine macrophages J774 were used in basal conditions to evaluate CEC mediated by passive diffusion; for total CEC cells were treated with 0.3 mM cpt-cAMP (Sigma-Aldrich, St. Louis, MO), which upregulates the aforementioned transporter. ABCA1-CEC was then evaluated as the difference in CEC between cells expressing ABCA1 and those in basal conditions.

Cells were seeded in 48-well plates at a density of 120000 cells/well. After 24 hours, cells were labeled with [1,2-3H]-cholesterol (PerkinElmer, Milan, Italy). After 24 hours from labeling, cells were subjected to an equilibration time in a culture medium containing 0.2% free fatty acid BSA (Sigma-Aldrich). During this phase, J774 underwent ABCA1 upregulation with a cAMP analog. The cells were then washed with PBS to remove dead cells and subsequently exposed to 2% (v / v) of the participants' sera for 4 hours. During radiolabeling and equilibration time was used ACAT inhibitor at a concentration of 2 µg/mL (Sandoz 58035, Sigma-Aldrich) to ensure that all cellular cholesterol is in the free form.

Serum CEC was expressed as a percentage of the ratio between the radioactivity released in the medium and the total radioactivity incorporated in the cells. A parallel set of cells was incubated with medium alone to provide a background efflux which was subtracted from the CEC values of the serum samples. The lipid-free normal human Apo-AI reference (Sigma-Aldrich) was used in each experiment to test cAMP-mediated induction of ABCA1 expression. In addition, a pool of normal human sera, the reference standard, was tested in each assay and its CEC value was used to normalize

the CEC values of the participating sera obtained in the experiments to correct the inter-assay variability.

Statistical analysis

Statistical analyses were performed using STATA 14. Every plasma sample was run in triplicate and average values and SDs were calculated for each percentage of efflux obtained. Basic statistical comparisons were then carried out using two-tailed Student's t-test for paired samples after confirming data normality through Shapiro-Wilk test. The association between parameters was assessed by means of Spearman correlation. Statistical significance was defined as $p<0.05$.
The crossover design was methodologically analyzed in two steps. First, we carried out a preliminary test to exclude the presence of carryover effects by using an unpaired t-test (after positive verification of homoscedasticity) on the two possible sequences of diets over time (Wellek and Blettner 2012). Second, we run a linear mixed-effect regression model, which estimates both the fixed-effects associated with diets (and the other factors) and the random-effects component associated with the repeated-measures structure of the data. The model allows to control for the correlation structure of the sample analyzed. The dependent variables are the changes in either ABCA1, Aqueous Diffusion, or total efflux between two time points. The explanatory variables are diet (0=Mediterranean, 1= Vegetarian), time (T1 and T2), and order of diets (0=MV, 1=VM), where the code 0 defines the reference category. The models control also for two covariates, namely sex and age. A robust estimator of variance was used to provide standard error estimates robust to various types of misspecifications, such as heteroskedasticity or the correlation structure (Huber 1967; White 1980).

Results

This project aimed to evaluate the functionality of HDL, expressed as CEC, mediated by passive diffusion (PD), total efflux and ABCA1, in sera of overweight omnivorous subjects.

In particular, the project intent was to evaluate the effects of a lacto-ovo-vegetarian (VD) diet compared to a Mediterranean diet (MD), both low-calorie, on HDL functionality in the same cohort of subjects at low-medium risk of CVD.

30 clinically healthy omnivorous subjects were enrolled, who were on a Mediterranean diet for three months and a vegetarian diet for three months, in a two-arm randomized controlled trial with cross-over modality.

Correlation between efflux pathways and clinical/biochemical parameters

In Table IIIa are reported the association at baseline, regardless the diet, between CEC mediated by each efflux pathway and some clinical/biochemical parameters of the subjects under examination. This analysis revealed the existence of a positive and significant correlation (rho = 0.462, p = 0.010) only between the ABCA1 transporter-mediated CEC and plasma adiponectin concentration. The correlations between the other clinical/biochemical parameters and the three outflow pathways were not statistically significant.

Clinical parameters	Mean±SD	Aqueous diffusion	Total efflux	ABCA1
Adiponectin	8.2±2.2	-0.314 p=0.091	0.212 p=0.261	**0.462** **p=0.010**
IL-17	10.6±9.0	-0.141 P=0.456	-0.084 P=0.659	0.030 p=0.874
TG	133.8±66.1	0.1270 p=0.5037	-0.069 p=0.719	-0.070 p=0.712
TC	221.0±33.6	0.023 p=0.902	0.098 p=0.607	0.035 p=0.856
HDL	59.0±16.6	0.264 p=0.159	0.321 p=0.084	0.106 p=0.579
LDL	135.2±32.1	-0.091 p=0.633	-0.069 p=0.717	-0.052 p=0.786
Glucose	89.4±13.7	-0.192 p=0.310	-0.226 p=0.230	-0.062 p=0.745

Table IIIa. Correlations between efflux pathways and clinical/biochemical parameters. Spearman rho coefficients. Significant associations are shown in bold.

CEC mediated efflux parameters and diets

The relationships between the different efflux pathways and MD or VD were analyzed. Table IIIb presents some descriptive statistics of ABCA1, aqueous diffusion and total efflux for the two diets, independently of the order of the diet. Statistical differences were found between the two diets for both ABCA1 and total efflux (t = 8,966, p <0.001 and t = 6,535, p <0.001, respectively), while the aqueous diffusion was not statistically different between the two treatments.

Diet	Mediterranean	Vegetarian	t	p-value
ABCA1	10.63±2.94	8.65±2.93	8.966	<0.001
Aqueous Diffusion	11.64±2.68	11.59±3.09	0.166	0.869
Total Efflux	22.24±3.24	20.24±3.14	6.535	<0.001

Table IIIb. CEC mediated efflux parameters by diet. Descriptive analysis

The crossover analysis to evaluate the effects of VD and MD on ABCA1, aqueous diffusion and Total Efflux consists of two distinct tests. The first phase consists in evaluating the possibility of the existence of a carryover effect in samples, while the second phase is carried out to adequately estimate the effects of the various explanatory variables on the three outflow pathways.

Table IIIc shows the results of the unpaired t-tests used to verify the existence of the carryover effect in the three crossover studies examined here.

Trial	t	p-value	DF
ABCA1	0.282	0.780	28
Aqueous Diffusion	0.045	0.964	28
Total efflux	0.323	0.749	28

Table IIIc: Carryover effect in the two crossover trials. Unpaired t-test

In all cases the test was not statistically significant (for ABCA1, t = 0.282, p = 0.780; for aqueous diffusion, t = 0.045, p = 0.964; for Total efflux, t = 0.323, p = 0.749), demonstrating the absence of

any entrainment effect for all the pathways. Tables IIId-e-f show the effects of different diets on ABCA1, aqueous diffusion, or total efflux once controlled for time and order of diets, as well as age and sex of the patients. For ABCA1, showed in Table IIId, VD seems to significantly decrease the concentration of ABCA1 by 4,148 units compared to the effect of MD. On the contrary, time and sequence do not appear to significantly influence the efflux mediated by ABCA1. Furthermore, was found evidence of a significant association between gender and ABCA1, because males showed a higher concentration of ABCA1 than females (+0.608, $p = 0.025$). Regarding the aqueous diffusion, showed in Table IIIe, none of the explanatory variables included in the model were statistically significant and, consequently, even the model itself did not produce any significant improvement in the fit of the model compared to the model with the constant included ($\chi 2 = 3.52$, $p = 0.621$). In contrast, the model for total efflux confirmed the pattern described above for ABCA1 (Table IIIf). The total efflux shows a statistically significant decrease in the presence of a VD (-4.238 units compared to the MD), while no effect was detected for time and sequence of diets. Likewise, age and gender did not have a significant effect on total efflux.

Variables	Coeff.	Robust SE	p-value
Vegetarian Diet	-4.148	0.271	<0.001
Time T2	0.160	0.537	0.765
Order V-M	-0.187	0.341	0.583
Age	-0.020	0.012	0.090
Male	0.608	0.271	0.025
Log-likelihod	-115.2		
Wald test	$\chi 2$=110.39	p<0.001	

Table IIId: Effects of diet on ABCA1. Mixed-effect linear regression

Variables	Coeff.	Robust SE	p-value
Vegetarian Diet	-0.136	0.414	0.743
Time T2	0.626	0.415	0.131
Order V-M	-0.051	0.325	0.876
Age	-0.005	0.010	0.628
Male	-0.399	0.351	0.256
Log-likelihod	-105.9		
Wald test	$\chi 2$=3.52	p=0.621	

Table IIIe: Effects of diet on Aqueous Diffusion. Mixed-effect linear regression

Variables	Coeff.	Robust SE	p-value
Vegetarian Diet	-4.238	0.512	<0.001
Time T2	0.830	0.513	0.105
Order V-M	-0.239	0.361	0.508
Age	-0.025	0.014	0.085
Male	0.210	0.314	0.504
Log-likelihod	-115.0		
Wald test	$\chi2=75.04$	$p<0.001$	

Table IIIf: Effects of diet on total efflux. Mixed-effect linear regression

The interaction between diets and time was tested as well in all trials, but no statistically significant outcome was found.

Discussion

Diet plays a fundamental role in maintaining the state of health and in determining cardiovascular risk. MD is considered the diet par excellence as it brings many benefits to health (Leitzmann 2014). MD helps fight the increase in blood triglycerides and decrease the cholesterol content, with benefits on the cardiovascular system, reducing the risk of strokes and heart attacks (Leitzmann 2014). MD is characterized by an abundant consumption of all food groups, including meat, poultry, and fish; this diet is rich in protein, fiber, omega 3, minerals and vitamins, and does not include industrial fats and sugars (Wellek and Blettner 2012). Furthermore, favoring the consumption of fruit, vegetables and legumes, all rich in antioxidants, MD increases the prevention of CVD (Leitzmann 2014).

Another dietary pattern that is gaining increasing popularity is VD. VD is the most common type of vegetarian diets, characterized by the exclusion of animal products such as meat, fish, and poultry in their fresh, preserved, and processed form; however, it allows the consumption of eggs and dairy products. This type of diet has many health benefits and helps in the prevention of many disorders, such as hypercholesterolemia, diabetes, and arterial hypertension (Dinu 2017). The absence of red meat, in particular, may have important effects on the lipid profile, which other dietary strategies are unable to produce. It has been suggested that, compared to omnivorous diets, vegetarian diets better achieve therapeutic goals for cardiovascular risk reduction, including the reduction of LDL and non-HDL cholesterol levels (Kahleova 2017). Furthermore, there is a relative reduction of HDL-C, total cholesterol, but without effects on triglycerides. LDL-cholesterol reduction alone has been shown to reduce coronary risk by 22% in people with average lipid levels (Kahleova 2017, Wang 2015).

A physiological mechanism used for the removal of excess cholesterol is RCT, that involves the removal of excess cholesterol from the peripheral tissues, through its incorporation into HDL lipoproteins, and the subsequent transport to the liver for elimination through the faeces (Favari 2013). The first stage of the RCT is the efflux of cellular cholesterol, that can occur in humans with active or passive transport mechanisms (Hill and McQueen 1997). HDLs have different ability to interact with cholesterol transporters and the effectiveness of HDL activity can be estimated by CEC measuring.

ABCA1-mediated efflux is one of the main cholesterol removal pathway, an important determinant of plasma HDL levels and a potent atheroprotective factor. This transporter is active in case of high cellular cholesterol levels and it helps macrophages to get rid of excess lipids (Li 2013).

It has recently been shown that CEC is inversely related to the incidence of cardiovascular risk, resulting in a better predictor of cardiovascular risk than HDL plasma concentration (Saleheen 2015). Therefore, there is growing evidence that measuring HDL function, assessed as CEC, can be considered a better predictor of coronary heart disease than measuring HLD-C levels (Khera 2011).

In light of these observations and on the basis of the knowledge related to the functionality of HDL to promote RCT, the aim of this third project was to evaluate the possible effect of MD and VD on HDL function in a group of thirty clinically healthy subjects with a low-to-moderate cardiovascular risk profile (21 F; mean age: 51.3 ± 9.7 years), who were randomly assigned to a 3-month MD or VD diet and then crossed.

Starting from the evaluation at baseline and regardless the diet was observed only a positive correlation (Table IIIa) between CEC-mediated by ABCA1 and plasma concentration of adiponectin (rho=0,462, p=0,010), no other significant correlation were found. Adiponectin is a protein hormone that modulates some metabolic processes, including the regulation of glucose and the catabolism of fatty acids. Adiponectin is secreted by adipose tissue into the bloodstream and its levels are inversely linked with the amount of fat in the body, for this reason obese produce lower levels of this hormone than normal-weight individuals (Fang and Judd 2018). Furthermore, it promotes the oxidation of fatty acids in the muscles, reduces their supply to the liver, the content of triglycerides and decreases the production of glucose in the liver (Fang and Judd 2018). Therefore, high concentrations of adiponectin found in study participants following adherence to these dietary types, confer beneficial effects thanks to its anti-atherogenic, anti-inflammatory and protective properties on cardiovascular risk.

Moving on to the different efflux pathways in relation to the type of diet, a significant difference was observed for both types of diets and CEC-ABCA1 mediated and Total-CEC ($t = 8.966$, $p < 0.001$ and $t = 6.535$, $p < 0.001$ respectively, Table IIIb). On the other hand, no significant differences were observed with passive diffusion (Table IIIb).

A crossover analysis was carried out to study the effects of the two dietary typologies on the three efflux pathways through two distinct tests. No entrainment effects were observed for the three outflow pathways (Table IIIc).

It was found that VD seems to decrease CEC-ABCA1 mediated compared to MD, while time and sequence of diets do not significantly influence the pathway. Furthermore, CEC-ABCA1 mediated was significantly higher in males than in females (+0.608, $p = 0.025$, Table IIId), demonstrating the existence of a significant association between gender and ABCA1.

Regarding HDL-CEC mediated by PD (Table IIIe), none of the explanatory variables included in the model were statistically significant.

Total efflux significantly decreased in the presence of VD (-4,238 units) compared to MD, while no effect was detected for time or sequence of diets (Table IIIf). Likewise, age and gender did not have a significant effect on this outflow pathway.

The role of serum CEC as an index of cardiovascular protection was first suggested by a study in which this variable showed an inverse relationship with carotid mid-intimal thickening, index of subclinical atherosclerosis, relative to plasma HDL-C levels in two distinct cohorts of subjects (Khera 2011). Based on this, several papers have been published, including our observation in which CEC in healthy subjects correlates inversely with vascular stiffness regardless of HDL-C levels (Favari 2013).

VD decreased CEC activity mediated by the ABCA1 transporter, but not CEC mediated by PD pathway, indicating a probable depletion of pre-β-HDL, small and low cholesterol-rich HDL, which modulate efflux activity through these pathways. In fact, according to a study carried out by Favari and colleagues, ABCA1-mediated cellular cholesterol efflux can be effectively driven not only by the poorly lipidized apolipoprotein AI (Favari 2009), but also by a small discoid particle containing phospholipids, defined as preβ-HDL. Furthermore, the reduction of CEC mediated by the ABCA1 transporter could further justify the predisposition of these subjects to cardiovascular risk, since, as evidenced by the literature data (Daimon 2003), the reduction of the activity of this transporter and the consequent activation of inflammatory processes characterizes the pathophysiology of atherosclerosis.

A possible explanation for the beneficial role of MD that has been seen in CEC in patient submitted to this type of diet, could be due to the lipid lowering effect promoted by the MD diet, that has been already documented (Tosti 2018).

Esposito and colleagues demonstrated how MD, rich in mono- and poly-unsaturated fats, allows an increase in HDL-C levels of 3 mg/dL (Esposito 2004). Furthermore, in a PREDIMED substudy Damesceno and collaborators highlighted how adherence to a Mediterranean-type diet leads to a less atherogenic lipoprotein profile thanks to the increase in large HDLs and to the reduction in LDL, especially small and dense LDLs (Damasceno 2013).

Another PREDIMED substudy was conducted to evaluate the effect of MD on HDL functionality (Hernáez 2017). A significant increase in the activity of PON1 and in the production of nitric oxide, promoted by HDL, was found following adherence to a MD with the addition of extra virgin olive oil compared to the low-fat diet (control diet). This data indicates a better antioxidant capacity of HDL and a stimulation of the vasodilatory capacity. It was also observed that the treatment with extra virgin olive oil increased the ability of HDL to esterify cholesterol and reduced the activity of CETP. Furthermore, adherence to a MD with the use of extra virgin olive oil has led to a change in the composition of HDL, with an increase in the surface PL content and a reduction in central TG (Hernáez 2017). The increase of PL in HDL favors an increase in the efflux of cellular cholesterol

towards HDL, thus potentially increasing the cholesterol eliminated by RCT (Andersen 2013; Tchoua 2010).

The study has some limitations that deserve to be discussed. Firstly, the short duration of the study and the limited number of participants allow only initial possible interpretation of the result. Studies with a longer duration and a larger population are needed to confirm these preliminary findings. Moreover, we acknowledge that this study was not originally designed for these analyses, but the results suggest that more studies are warranted to assess possible differences in responses to VD and MD. A strength of our study, on the other hand, is the high compliance of the participants to the assigned diets. Furthermore, the crossover design allowed us to minimize inter-individual variability, as the participants served as their own control.

Part IV: Antibodies anti Apo-AI as a regulator of triglyceride metabolism and a possible contributor to NAFLD development

Aim

Anti Apo-AI antibodies represent a plasma factor capable of influencing lipid metabolism, as well as being a biomarker of cardiovascular risk in primary and secondary prevention (Antiochos 2016; Vuilleumier 2010). Translational studies demonstrate a role of these autoantibodies in mediating atherosclerotic and atherogenic processes, and they are also responsible for myocardial necrosis and death in mouse models, through toll-like 2,4 receptors (TLR 2,4) and CD14 signaling (Montecucco 2015; Pagano 2016).

Studies conducted by different research groups show an inverse association between the levels of anti Apo-AI IgG and total cholesterol, HDL, LDL (Antiochos 2016; Bridge 2018; Pagano 2016; Quercioli 2012). This data demonstrates a possible ability of these antibodies to affect the metabolism of cholesterol, in addition to pro-inflammatory and pro-thrombotic effect (Montecucco 2011; Pagano 2012, 2016).

The molecular mechanisms responsible for the changes induced by the anti Apo-AI antibodies are partially known. Previous study by Pagano et al (Pagano 2019) shown that anti Apo-AI increase the expression of important regulators of the cholesterol homeostasis such as HMGCR, LDL-R and SREBP-2. Following the upregulation of these key proteins an increase in the intracellular uptake of LDL is observed leading to the foam cell formation (Pagano 2019). These data confirm an alternative lipid uptake mechanisms already described (Moore 2005; Ye 2009) that may contribute to macrophage cholesterol ester accumulation different to the canonical one mediated by the uptake of oxLDL through scavenger receptor (Endemann 1993).

The aim of this last project, carried out at the Department of Medicine, of the University of Geneva, under the supervision of Professor Nicolas Vuilleumier, was to investigate the effect of Anti Apo-AI antibodies on other genes involved in lipid metabolism, i.e. sterol regulatory element binding protein 1 (SREBP-1), fatty acid synthase (FASN) and glycerol phosphate acyltransferase 1 (GPAT1) in hepatocytes in order to find a link between anti Apo-AI and fatty liver.

Previous and still unpublished data by Vuilleumier’s laboratory reported that Anti Apo-AI antibodies increase the lipid droplet content even in HepaRG cells, an hepatic cell line. We decided to better investigate the above-mentioned pathway.

SREBP-1 is a transcription factor, regulator of the main genes that control cellular lipid homeostasis (Bertolio 2019). In vertebrates, SREBP-1, together with SREBP-2, is translocated from the endoplasmic reticulum to the Golgi in response to cholesterol deprivation. Once they get to the nucleus, they induce the expression of genes mainly involved in the biosynthesis of sterols and fatty acids (Bertolio 2019). On the other hand, fatty acid synthase (FASN) is a key enzyme in the endogenous lipogenesis pathway, involved in the catalysis of the synthesis of long-chain saturated FA palmitate starting from acetyl-CoA and malonyl-CoA, using NADPH as a reducing agent

(Schroeder 2021). While glycerol phosphate acyltransferase (GPAT) is a key gene in the fate of the Acyl-CoA fatty pool at the cellular level, in the outer mitochondrial membrane in the liver (Takeuchi and Reue 2009). An overexpression of the gene leads to an increase in the uptake of exogenous fatty acids in TAGs and phospholipids and a reduction in β-oxidation (Takeuchi and Reue 2009).

To try to better understand the involvement of this autoantibodies in a possible involvement of fatty liver process the aim of this study was: 1) to test the effect in gene involved in lipid metabolism, such as SREBP-1, FASN and GPAT1, 2) to test the effect on pro-inflammatory cytokines production by liver cells, such as IL-6, IL-8 and TNF-a, and 3) evaluate whether by blocking SREBP-1 and the TLR2-4 and CD14 receptors there is a reduction in the inflammatory response.

Materials and Methods

Cell line and culture conditions

Cells manipulation took place in sterility, using a vertical laminar flow hood (Safeflow 1.2 Bioair). The glassware was previously sterilized in a dry oven for 4 hours at 180 °C, while the plastic materials used were purchased already sterile.

William's E medium (Gibco) was enriched with 1% of a solution of Penicillin (100 U/ml) Streptomycin (100 μg/ml) (Life technologies), 10% of L-Glutamine (Gibco), 0.00125% of a human insulin solution (10 μg/ml) (Sigma Aldrich), 0.05% of hydrocortisone 21-hemisuccinate sodium salt (Sigma Aldrich). The medium was stored at 4 ° C in sterile conditions in order to avoid alterations and contamination. If necessary, 10% of HyClone Calf Serum (Cytiva life sciences) was added, and to wash cells was used DPBS from Gibco. The cell culture was kept in incubator (RS Biotech, UK) at 37 °C at 5% CO_2 with a humidity of 90-95% and the morphological observation of the cells was carried out using an optical microscope (Olympus CKX41). Multiwell culture plates for western blot and MSD MULTI-SPOT assay system are from Costar, supplied by Corning Incorporated. The easy grip tissue culture dishes used for the enzymatic kinetic are from the Falcon company.

The experiments were conducted using HepaRG human liver cells. The cells were flasked at a density of 2.5×10^4 cells/cm^2, kept in culture medium for 2 weeks, changing it twice a week. After two weeks, the cells were subjected to a differentiation period of 2 weeks in William's E medium with 1.8% of DMSO Hybra-Max (Sigma Aldrich).

Protein extraction and western blot analysis

To estimate the effect of Anti Apo-AI on SREBP-1, SREBP-2, FASN and GPAT1 proteins, differentiated HepaRG cells were seeded in 6-well plates at a density of 255000 cells/well in William's E medium enriched with 10% HyClone Calf serum and 1.8% of DMSO Hybra-Max.

After 24 from sowing, cells were treated with the culture medium alone, or with goat Anti-human Apo-AI (Academy Bio-Medical Co) at a concentration of 40 μg / ml or with goat IgG (Meridian Life Science) at a concentration of 40 μg /ml. After 24 hours from the treatment, cells were placed on ice and washed in cold DPBS, and 100 μl/well of RIPA buffer 1X with a cocktail of phosphatase inhibitors and 1% NP40 was added. Cells remained on ice with RIPA buffer for 20 minutes. Subsequently to detach cells, was used a cell scraper, transferring the content of each well into tubes, leaving them on ice for another 10 minutes. At this point the lysates are centrifuged at 140000g for 20 minutes at 4 ° C and the supernatants were transferred to other tubes and stored at -20 °C. To proceed with Western Blot analysis, protein quantification took place using micro BCA protein assay kit (Thermo Scientific). 20 μg per lane were loaded on 4-15% pre-fabricated polyacrylamide gel (Biorad) inside a protein electrophoresis chamber (Biorad), in presence of a running buffer consisting

of deionized water and 10% of 10XTris-glycine- SDS buffer (Biorad). The electrophoretic run took place with a constant voltage of 150 Volts. After the electrophoretic run, the samples were transferred onto extra thick paper (Biorad) in the presence of transfer Buffer containing distilled water, 10% of 10XTris-glycine-buffer (Biorad) and 20% of methanol by trans-blot SD cell of semi-dry transfer (Biorad) at 150 mA for 1 hour. To reduce the possibility of non-specific binding the membrane was blocked by using 5% non-fat milk powder in T-TBS for one hour at room temperature. After this step, the membrane was incubated under agitation with each primary antibody in 5% milk in T-TBS. The mouse anti-SREBP2 antibody (Cayman Chemicals) was used 1:1000 overnight at 4 °C, the mouse anti-SREBP1 antibody (NovusBio) was used 1:1000 overnight at 4 °C, the rabbit anti-FASN antibody (ABCAM) was used 1:5000 overnight at 4 ° C, rabbit anti-GPAT1 antibody (NovusBio) was used 1: 1000 overnight at 4 ° C, finally mouse anti-Tubulin antibody (Santa Cruz) was used as control diluted 1: 2000 1 hour at room temperature. As an alternative to anti-tubulin antibody, mouse anti-actin antibody diluted 1:5000 (ABCAM) was also used as a control. After 3 washes in T-TBS each antibody was incubated with secondary anti-mouse antibody (1: 4000 for SREBP-2, SREBP-1, tubulin and actin, produced by Dako) or anti-rabbit (1: 4000 for both GPAT1 and FASN, produced by Dako) for 1 hour at room temperature under gentle agitation.

The detection of the proteins on the membrane was performed by incubation for 5 minutes with BM chemioluminescence blotting substrate (Roche Dignostic) and then by impression on the radiographic plate, developed with CURIX60 AGFA processor film. The signal was quantified by Image J software. Results are expressed in arbitrary units.

Regarding the study on the kinetics of protein expression following treatment at different times with Anti Apo-AI Antibody, the HepaRGs were plated in 35 mm easy grip tissue culture dish at a density of 1800000 cells/dish.

After 24 hours from sowing, cells were treated with Anti Apo-AI at a concentration of 40 μg/ml for 0h (treated only with culture medium), 3h, 6h, 9h, 12h and 24h. The treatment was stopped by washing in DPBS and subsequently the cells were stored at -80 ° C. The protocol previously described was used for the western blot analysis.

Evaluation of TLR-2,TLR-4 and CD-14 expression

The Fluorescence-activated cell sorting (FACS) method was used to evaluate the expression of TLR-2, TLR-4 and CD-14 in HepaRG cells.

Briefly, cells were seeded at a density of 300000 cells per well in 96-well conical bottom plates (Biosigma). Then the 96-well plate containing the seeded cells was centrifugated for 5 minutes at

1000 rpm to carry out a first wash. After removing the supernatant medium, the cells are left in 100 μl of PBS-1% BSA for 15 minutes.
At this point the cells were centrifuged for 5 minutes at 1000 rpm to eliminate the washing liquid and then the cells were incubated for 45 minutes at 4 °C in the dark in 100 μl of PBS-1% BSA containing 2.5 μl of the antibody anti-TLR-2, anti-TLR4 or anti-CD14, labeled with APC probe (Biolegend), or with 2.5 μl of the IgG ctrl labeled with APC o FITC probes. Two wells also cointained cells incubated with 100 μl of PBS-1% BSA plus 2,5 μl of culture medium (untreated cells).
After 45 minutes, the plate was centrifugated for 5 minutes at 1000 rpm and then was removed the supernatant and continue with 3 washes in 100 μl of PBS-1% BSA. At this point, the cells labeled with antibody, linked to the probe, were resuspended in 300 μl of PBS-1% BSA and transferred to Eppendorf to continue with the analysis.
The fluorescence intensity was analysed with the BD Accuri ™ C6 Plus Flow Cytometer and a minimum of 0.3 millions cells/sample was acquired. Analysis was preformed using FlowJo software (Treestar).

Cytokines assessment after Anti Apo-AI treatment

After 24h treatment with Anti Apo-AI IgG or IgG control or in presence of culture medium (basal condition), Interleukin 6 (IL-6), Interleukin 8 (IL-8) and Tumor Necrosis Factor (TNF)-α were measured using the V-Plex Proinflammatory Panel 1 kit from MesoScale Discovery (MSD) platform (Rockville, MD, USA) on the SQ120 instrument, following the producer instruction.

Cytokines assessment after SREBP-1 silencing and blocking of TLR2/4 and CD14 receptors

The Trasnfecting Stealth siRNA kit (Invitrogen) was used into HepaRG cells in presence of lipofectamine RNAiMAX for gene silencing of SREBP-1.
A reverse transfection protocol was performed, using 96-well plates (Corning). The transfection process started by adding 150 μl/well of each solution containing respectively one of the 3 siRNAs (siRNA1, siRNA2, siRNA ctrl): 3 μl siRNA (1μM) + 139.5 μl OPTI-MEM 1X (Gibco) and 7.5 μl of Lipofectamine RNAiMAX diluted 1:10 in OPTI-MEM 1X.
The treatment was carried out by adding 30 μl of OPTI-MEM 1X alone for the control condition or 30 μl of the different solutions with siRNAs. Subsequently, the HepaRGs were sown on top of these solutions at a density of 15000 cells/well using William's Medium E without antibiotics in a volume of 130 μl/well.

48 hours after transfection, after removing the medium containing the siRNAs, HepaRG were treated for 1 hour with 50 µl of a solutions containing or not blocking antibodies to TLR-2, TLR-4 and CD14 receptors. Anti TLR-4, anti TLR-2.1, anti TLR-2.5 and anti CD14 antibody (BioLegend) were used at a concentration of 20 µg / ml.
1 hour after the treatment described above, cells were treated for 24h with Anti Apo-AI antibodies (40 µg/ml), or with control IgG (40 µg/ml) or with the culture medium alone.
Interleukin 6 (IL-6), Interleukin 8 (IL-8) and Tumor Necrosis Factor (TNF) -α were measured, as previously described.

Statistics

For the evaluation of the pro-inflammatory effects by different treatments the results were expressed as median with interquartile range and range. P-value was calculated with Mann-Whitney test. Statistical significance was defined as $P < 0.05$. All the statistical analysis were performed using GraphPad 9.0.1.

Results

Effect of Anti-Apo-AI IgG treatments on protein expression in HepaRG cells

It has been shown that anti-Apo-AI IgG in macrophages lead to an increase in the lipid content through the induction of a key protein for lipid homeostasis, known as SREBP-2 (Pagano 2019). Previously and still unpublished data by Pagano demonstrated that even in another cellular model, HepaRG liver cells, the treatment with anti-Apo-AI IgG involves a significant increase (***p= 0.0002) in the number of lipid droplets compared to the condition with the culture medium alone and compared to control IgG (### p = 0.0008) as shown in figure IVa.

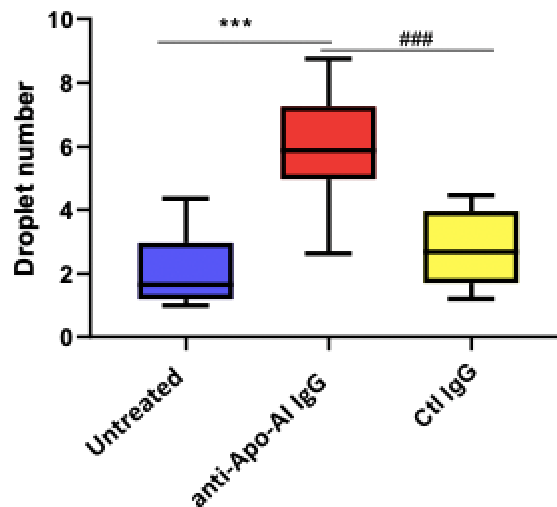


Figure IVa: evaluation of lipid droplet number respectively in untreated HepaRG cells, in cells after anti-Apo-AI IgG treatment, and after Ctrl IgG treatment. Results are expressed as median with interquartile range, P-value calculated with Mann-Whitney test. *** P=0.0002, ### P=0.0008. N=3.

We wanted to evaluate in HepaRG cells by Western Blot analysis the effect of anti-Apo-AI IgG on some proteins involved in the metabolism of triglycerides, in particular SREBP-2 (mature form), SREBP-1 (mature form), FASN and GPAT1.

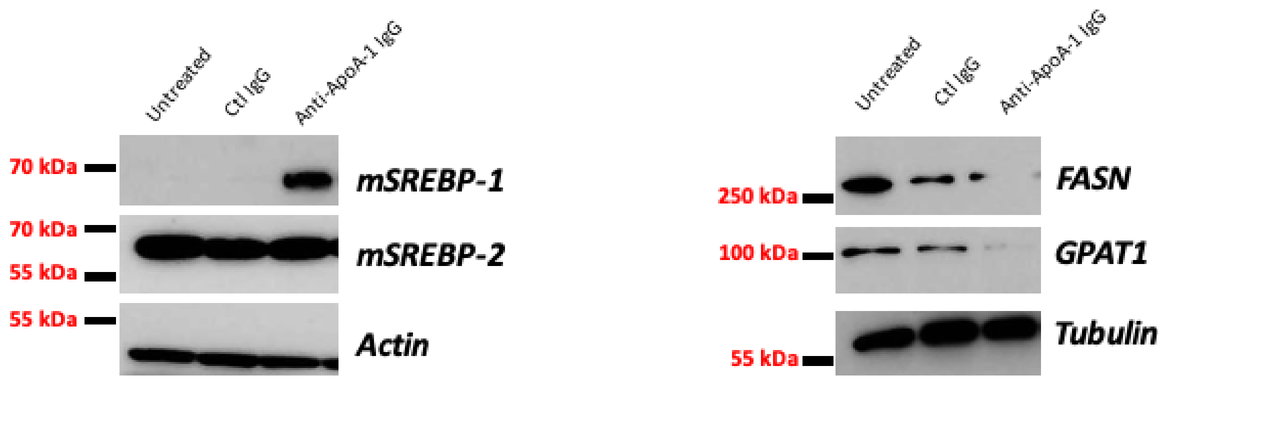


Figure IVb: Western Blot assay was performed using HepaRG cells lysates, untreated or treated with Ctrl IgG or with Anti-Apo-AI IgG. The assay was performed as previously said in Material and Methods section.
On the left Actin was used as control, while on the right Tubulin. mSREBP-1 and mSREBP-2 indicates SREBP-1 and SREBP-2 in mature form.

As shown in Figure IVb left panel, 24 hours treatment of HepaRG cells with anti-Apo-AI IgG in doesn't induces the expression of mSREBP-2, as previously demonstrated in macrophages but its expression remains constant in all conditions. Conversely mSREBP-1 expression is induced only in the presence of anti-Apo-AI IgG, while is not expressed in non treated or ctl IgG treated cells. In addition, on the right panel, we observe that only the anti-Apo-AI IgG treatment induce a decrease in FASN and GPAT1 expression.
At this point it was decided to evaluate the kinetic trend of the different protein expression under study at different time points, from 0 to 24 hours, after anti-Apo-AI IgG treatment, precisely after 3h, 6h,9h,12h and 24h.
As can be seen from Figure IVc, treatment with anti-Apo-AI IgG leads to a peak of SREBP-1 expression at 6-9h, which lasts up to 24h. On the contrary, the expression of FASN and GPAT1 increases at 3-6 hours and then gradually decreases up to 24 hours of treatment.

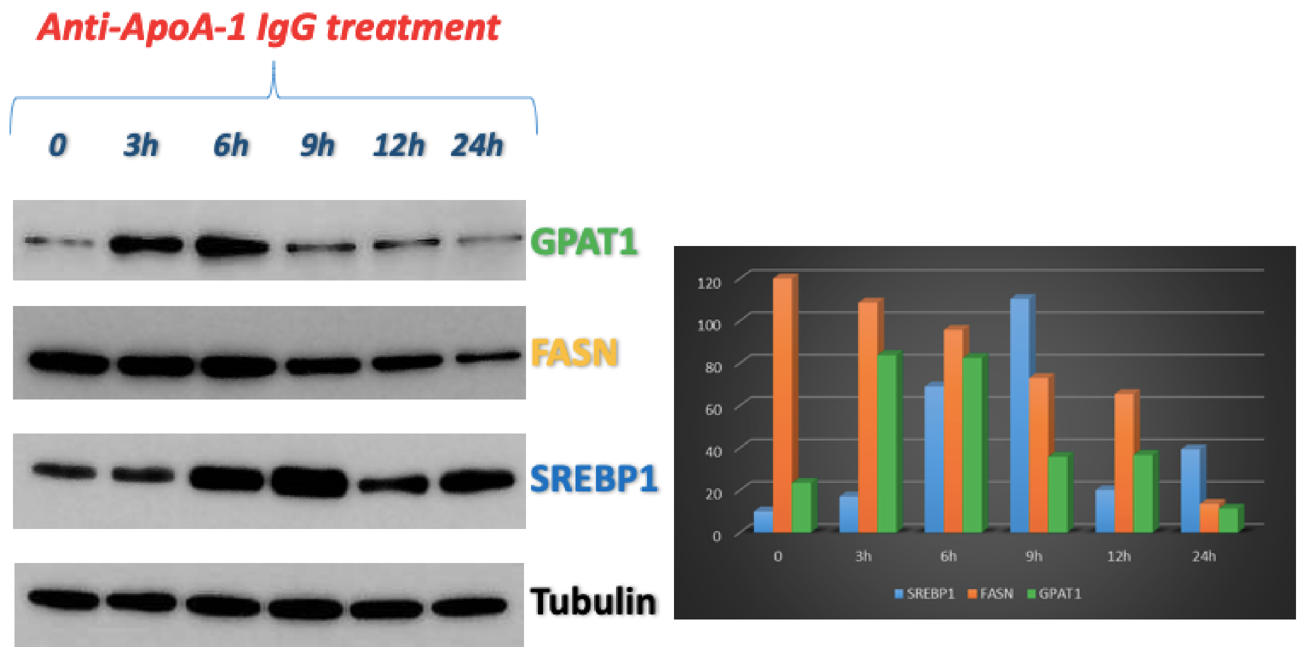


Figure IVc: Western Blot assay was performed using HepaRG cells lysates, untreated (0h) or treated with Anti-Apo-AI IgG at different time point. The assay was performed as previously said in Material and Methods section. Tubulin was used as control. Signal quantification of proteins expression was measured by ImageJ software. Signal quantification results were expressed as the ratio between in study proteins and the correspondent Tubulin signal and data are expressed as mean ± SD (n = 3).

Evaluation of TLR-2,TLR-4 and CD-14 expression in HepaRG

The expression of TLR-2, TLR-4 and CD14 in HepaRG was evaluated using FACS technique as described in the materials and methods paragraph.

As can be seen from Figure IVd HepaRG cells seem to express only TLR-2 receptor. Regarding TLR-4 and CD-14, cells marked with specific antibody against these receptors have an area that overlaps that of the untreated cells and cells marked with isotype control antibody (Isot Ctrl), thus indicating a lack of expression of the two receptors by HepaRG.

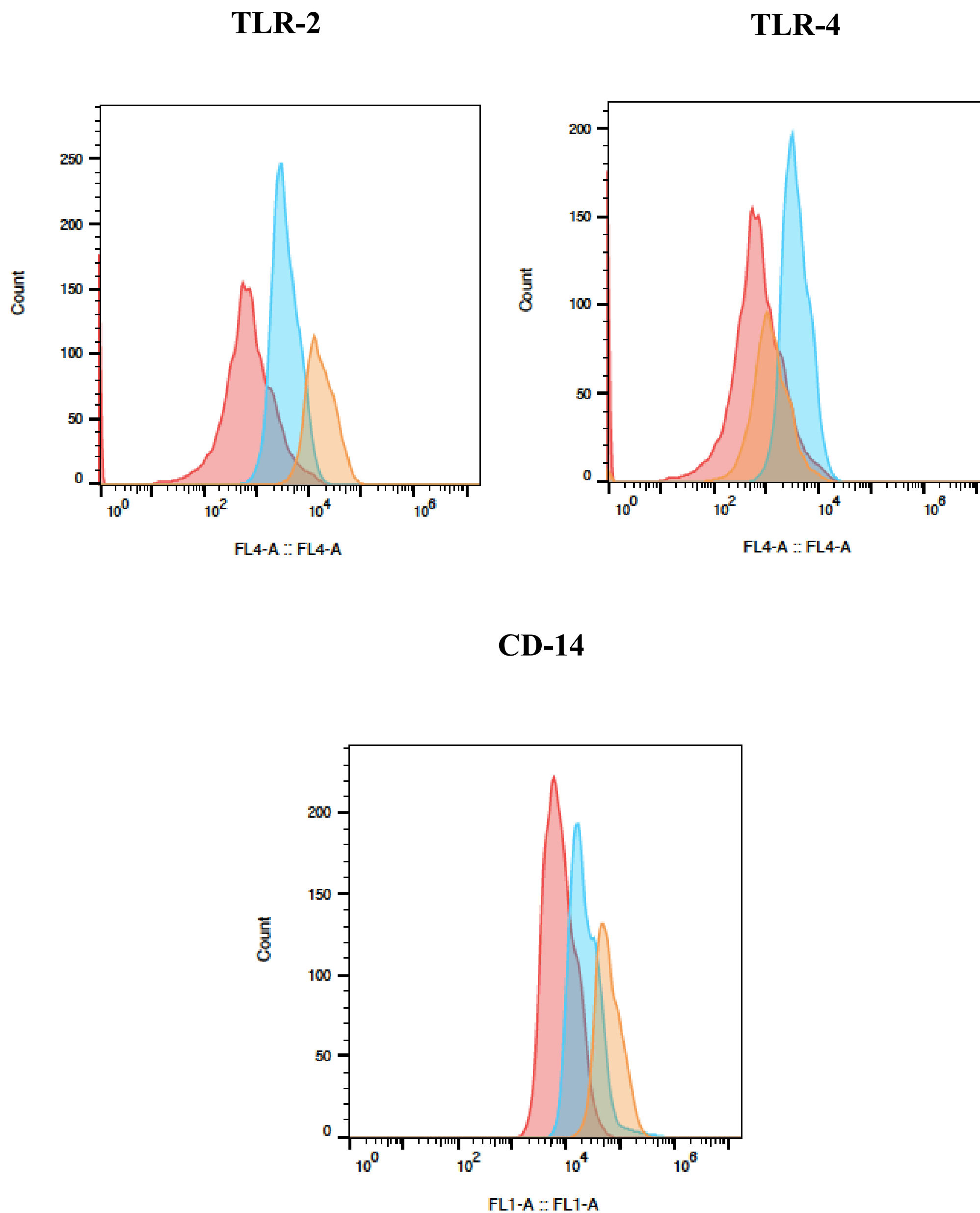


Figure IVd: FACS analysis was performed to evalutate expression of TLR-2,TLR-4 and CD-14 in HepaRG cells. Cells were treated for 45 minutes with Anti-TLR-4, Anti-TLR-2 or Anti-CD14 (show in orange). Cells were treated also with the specif IgG control (isot Ctrl, shown in light blue) or with only culture medium (Untreated shown in red).

Evaluation of the potential pro-inflammatory effect of Anti-Apo-AI IgG

Subsequently, was analyzed the effect of Anti-Apo-AI IgG on the production of pro-inflammatory cytokines, such as IL-6, IL-8, TNF-α in HepaRG cells.

As can be seen from figure IVe the presence of Anti-Apo-AI IgG induces a significant increase in the production of IL-6, IL-8, TNF-α respect to the basal condition and to the condition with IgG control (all P = 0.0079).

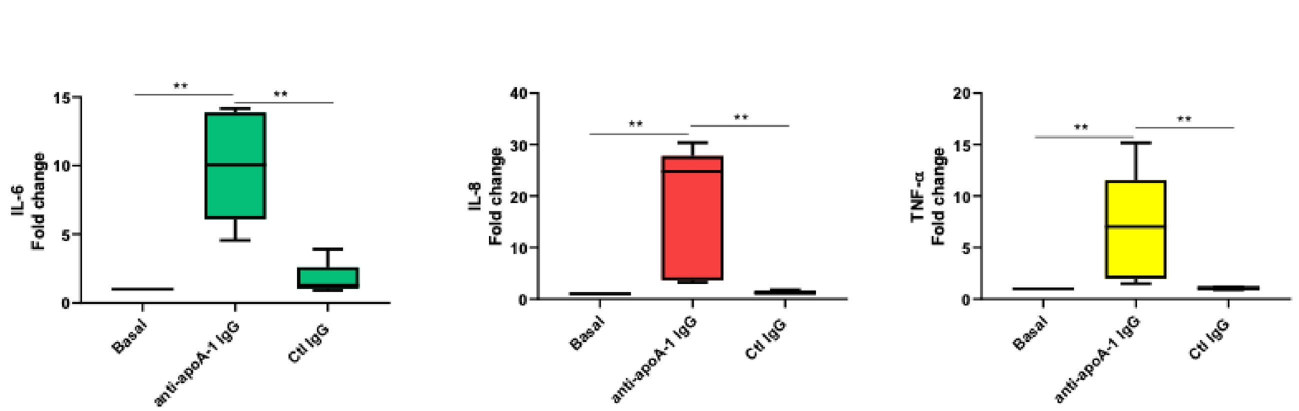


Figure IVe: MSD Multi-spot assay system was performed to detect IL-6, IL-8 and TNF-α levels in HepaRG cells in basal condition or after Anti-Apo-AI IgG treatment, or Ctrl IgG treatment. Results are expressed as median with interquartile rang and range. P-value calculated with Mann-Whitney test. ** P=0.0079. N=5

Then was evaluated if, silencing SREBP-1 for 48 h, the treatment with Anti-Apo-AI IgG resulted in a lower inflammatory response. As can be seen in Figures IVf, co-treatment with siSREBP-1 and Anti-Apo-AI IgG does not lead to a decrease in any of the inflammatory cytokines analyzed. Instead blocking the TLR-2 receptor with anti TLR-2 antibodies leads to a decrease in the production of IL-8, IL-6 and TNF-α (respectively P=0,1, P=0,02, P=0,05).

Co-treatment with anti-TLR-4 and anti-CD-14 together with Anti -Apo-AI IgG does not lead to a decrease in the production of these cytokines.

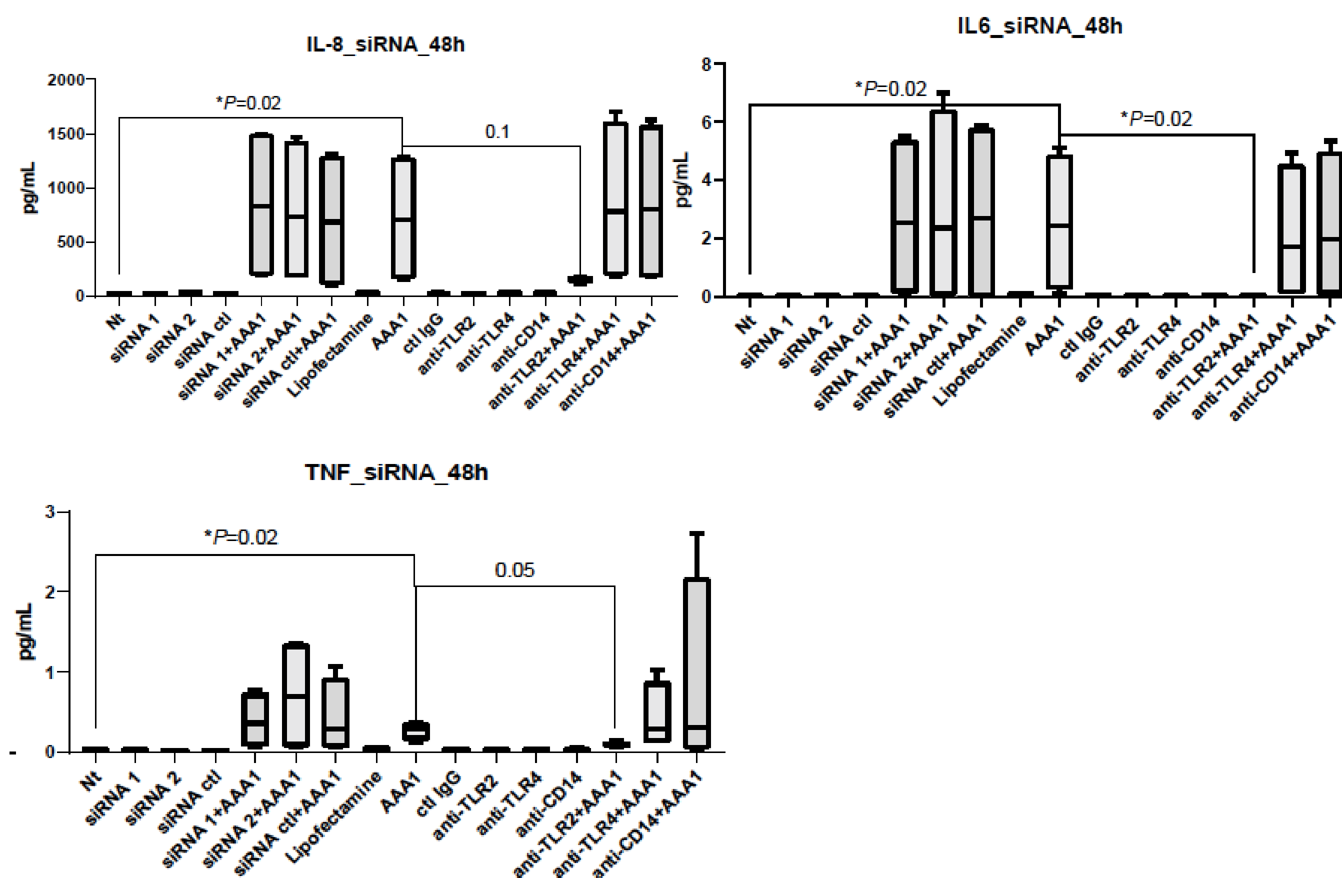


Figure IVf: MSD Multi-spot assay system was performed to detect IL-6, IL-8 and TNF−α levels in HepaRG cells. Cells were treated with culture medium (Nt), or with siRNA1, siRNA2, siRNActrl in presence or absence of Anti-Apo-AI IgG (AAA1) treatment, with lipofectamine, with Anti-Apo-AI IgG and IgG ctrl alone, or with anti-TLR-2, anti-TLR4, anti-CD14 alone or in presence of Anti-Apo-AI IgG treatment.
Results are expressed as median and IQR. P-value calculated with Mann-Whitney test.

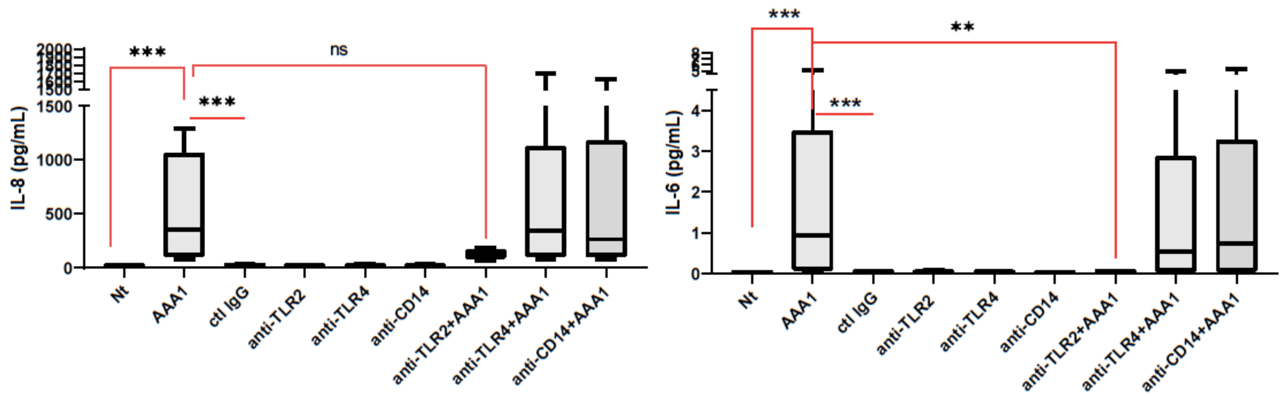
By analyzing the treatment for 24h with anti-TLR-2, anti-TLR-4 and anti-CD14 antibodies (figure IVg), a significant reduction of the inflammatory stimulus can be observed in the presence of Anti-Apo-AI IgG for IL-6 and TNF-α, in presence only of co-treatment with anti-TLR-2 (** P = 0.007 and * P = 0.016, respectively).

These results let us to suggest that the anti-ApoAI proinflammatory response is mediated by TLR-2.

***P=0,0002

***P=0,0002

**P=0,007



***P=0,0002

*P=0,016

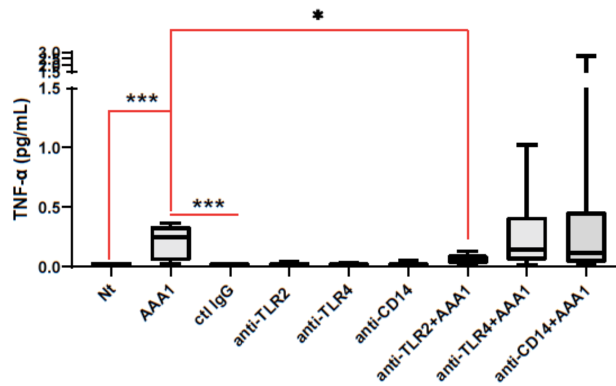


Figure IVg: MSD Multi-spot assay system was performed to detect IL-6, IL-8 and TNF-α levels in HepaRG cells. Cells were treated with culture medium (Nt), with IgG ctrl, with Anti-Apo-AI IgG (AAA1), or with anti-TLR2, anti-TLR4, anti-CD14 alone or in presence of AAA1.

Results are expressed as median and IQR. P-value calculated with Mann-Whitney test.

Discussion

Anti-Apo-AI antibodies are a known plasma cardiovascular risk biomarker. Studies show the presence of high levels of Anti-Apo-AI IgG in plasma in the presence of cardiovascular-based diseases (Keller 2012; Montecucco 2011; Vuilleumier 2010), but also in inflammatory/immune-based disorders such as SLE and APS (Haque 2008; Manzi 1997; Marai 2008; Salmon and Roman 2008).

The presence of high levels of Anti-Apo-AI IgG in plasma would lead to an increased risk of atherogenesis (Vuilleumier 2014). A first hypothesis on the mechanism of action of these antibodies consisted in a possible alteration in the functionality of HDL (Ames 2010; Batuca 2007). In fact, an inverse relationship with CEC mediated by passive diffusion was identified in a recent study from 2019 (Vuilleumier 2019).

These antibodies seem to influence cholesterol homeostasis. There is an inverse relationship between the levels of Anti-Apo-AI IgG and the levels of HDL, LDL and total cholesterol (Antiochos 2016; Bridge 2018; Montecucco 2011; Quercioli 2012).

In addition, a previous analysis has shown how these autoantibodies are able to induce an accumulation of lipids at the macrophage level, leading to an increase in the expression of SREBP-2, a protein involved in lipid metabolism, resulting in an increase in cholesterol synthesis and in an increase in LDL-R expression with consequent greater cholesterol uptake (Pagano 2019).

This increase in lipid droplets, found at the macrophage level, was found in another cell. Previously Pagano demonstrated how treatment of HepaRG cells with anti-Apo-AI antibodies for 24h leads to an increase in the number of lipid droplets compared to the control IgG condition and to the untreated condition.

In this study the aim was confirm this data and evaluate the effect of these autoantibodies on other proteins involved in the metabolism of triglycerides, such as SREBP-1, GPAT1 and FASN to further explore a possible link between the presence of high Anti Apo-A1 IgG levels and fatty liver leading to NAFLD that is known to have an impact on the development and progression of cardiovascular disease.

By Western Blot it has been shown how the treatment with Anti-Apo-AI IgG induces the expression of SREBP-1, while the expression of SREBP-2 is maintained unaltered. FASN and GPAT1, on the other hand, are reduced by treatment with Anti-Apo-AI IgG, compared to the other conditions.

At this point, the kinetic trend of these enzymes was evaluated. SREBP-1 is increased at 24h, while FASN and GPAT1 after a peak at 3/6h, decrease up to 24h. These results suggest that a negative feedback regulatory circuit turn off these enzymes probably due to the lipid overload found in the cells after 24h anti-Apo-A1 treatment.

Studies have demonstrated the key role of the TLR2-4/CD14 complex for the pro-inflammatory, pro-thrombotic and pro-arrhythmogenic cytokine-dependent biological properties attributed to anti-ApoA-1 IgG (Pagano 2012, 2016; Vuilleumier 2010). We wanted to evaluate the expression of these receptors in HepaRG. From the analysis by FACS it was observed that this cell line expresses only the TLR-2 receptor.

It was also found that treatment with Anti-Apo-AI IgG induces an increase in the production of pro-inflammatory cytokines by hepatocytes, such as IL-6, IL-8 and TNF−α which would better support anti-ApoA1 as a possible contributor to NAFLD development.

Then by blocking SREBP-1 with specific siRNA, it was observed how the treatment with Anti-Apo-AI IgG involves the maintenance of the inflammatory response even in absence of SREBP-1 expression. On the other hand, treatment with Anti-Apo-AI IgG together with anti-TLR-2 antibodies leads to a reduction of the inflammatory response, which does not occur in case of treatment with anti-TLR-4 or anti-CD14 antibodies.

Therefore, this study shows that Anti-Apo-AI IgG would promote an inflammatory response mediated by the TLR-2 receptor, instead the activation of SREBP-1 would not be necessary.

Final conclusions

Cardiovascular disease has several risk factors, among which cholesterol metabolism plays an essential role. LDL as a result of structural changes become more pro-atherogenic and tend to accumulate in macrophages, where they give rise to the formation of foam cells, which form the basis of atheroma. This process is opposed by the reverse transport of cholesterol (RCT), in which the first stage is the outflow of cholesterol from the cell. This step, promoted by the interaction of different subclasses of HDL with membrane transporters, allows to counteract the accumulation of cellular cholesterol, thus constituting an anti-atherogenic defense mechanism. To evaluate the ability of HDL to promote cellular cholesterol efflux, there is a parameter, called CEC (cholesterol efflux capacity). Following the failure to reduce CVD risk following treatment with drugs capable of increasing HDL levels, the role of HDL quality as a determinant of CV risk has been hypothesized. Several experimental evidences have shown that CEC, as a HDL functionality parameter, can represent a potential biomarker correlated inversely to CVD risk (Adorni 2021).

In cholesterol homeostasis, not only the amount of cholesterol that escapes through the efflux is important, but also the amount of cholesterol that enters the cell. For this, a parameter has been formulated that evaluates the ability of serum lipoproteins to transfer cholesterol to the macrophage, called CLC (cholesterol loading capacity). CLC is therefore a direct parameter for the evaluation of serum pro-atherogenic activity.

For this reason, in the first project of this thesis we wanted to analyze CLC in a group of patients at high risk of CAD, suffering from high levels of Lp (a) and subjected to apheresis. In fact, in addition to LDL, another pro-atherogenic plasma factor is Lp (a). This lipoprotein, similar to LDL, is more prone to oxidation and, therefore, more able to increase foam cells formation. Lp (a) presents gene structures defined Kringles, of which KIV2 is the determining factor, which, by varying in number of copies, involves a variation not only in the quantity but also in the size of Lp (a).

In this study it was observed that in the presence of small Lp (a), with a lower number of gene copies of KIV2, there is a greater number of these particles, while in case of larger Lp (a) the number of particles is reduced.

CLC was confirmed to be an index to evaluate CVD risk. In patients suffering from high levels of Lp (a) and CAD, a direct association between the size of Lp (a) and CLC could be observed. The larger the size of Lp (a), the lower the CLC. Therefore larger particles of Lp(a) would be associated with a lower risk of formation of foam cells.

Through this study, therefore, it was possible to ascertain how genetic variants of LP(a) are capable of positively or negatively influencing CLC and consequently potentially the risk of atheroma formation.

In addition to the influence of genetic variants, metabolism also plays an essential role in determining CVD risk. Therefore, in the second project, the presence of changes in CEC and CLC in patients suffering from two forms of NAFLD, one on a genetic basis and one on a metabolic basis, was evaluated as a possible cause of the different CV risk.

NAFLD is a multifactorial disorder caused by excessive amount of TG in the liver. NAFLD can be on a genetic basis, in the presence of alterations in genes involved in the metabolism of TG, or on a metabolic basis in the presence of metabolic syndrome or lipid metabolism disorders.

In this study, an alteration of CEC and CLC was observed in patients with NAFLD on a metabolic basis, not found in patients with the genetic form. Although the amount of fat in the liver was the same, CEC was not reduced in patients affected by the disease on a genetic basis. This confirms that the presence of an excess of fat in the liver is not a sufficient condition to determine these imbalances, on the contrary an alteration of the metabolism would seem necessary. Probably CEC alteration is caused by variations of the preβ-HDL responsible for the ABCA1-mediated efflux, the main path actively contributing to cholesterol efflux. In fact, this subclass of lipoproteins was found to be reduced in the group affected by metabolic disorders compared to the group with genetic variant. This decrease in CEC was also more marked in the presence of metabolic syndrome. In addition to this, CLC was also impaired in patients with metabolic disorders, which increased significantly compared to the group with genetic disorders. This increase in CLC was also more consistent in presence of MetS.

The alteration of these two parameters in patients with metabolic NAFLD, not found in those with genetic alterations, represent a possible cause responsible for the greater CV risk, underlying the importance of the lipid metabolism in the evolution of this pathology. In addition, this study shows the importance of classifying the forms of NAFLD to identify the potential CVD risk.

While genetics and metabolism influence cardiovascular risk, diet appears to be an approach for its management.

The Mediterranean diet (MD) brings several benefits to the cardiovascular system, in part thanks to the reduction of triglycerides and cholesterol. Even the vegetarian diet (VD), characterized by the exclusion of animal products, leads to positive effects on the lipid profile, thus helping to prevent various diseases, such as hypercholesterolemia. In this project we wanted to analyze whether adherence to a dietary approach, such as MD and VD, was able to influence the functionality of HDL, assessed as CEC, within a group of healthy subjects at medium-low cardiovascular risk assigned to one of the two diets and then crossed. It was observed that the VD significantly reduced the total efflux compared to the MD, while no change was observed instead for the CEC mediated by passive diffusion. VD also reduced the mediated CEC-ABCA1 compared to MD. This decrease in CEC

mediated by ABCA1, not observed in the case of passive diffusion, indicates a probable reduction in preβ-HDL. The benefits promoted by MD could be due to the lipid-lowering effects of the diet, which would be able to promote anti-atherogenic effects by increasing large HDL and reducing LDL. Furthermore, studies report the ability of MD to improve the antioxidant properties of HDL and to stimulate vasodilatory capacities leading to benefits on cardiovascular health.

In the last part of this thesis study we wanted to investigate a possible mechanism of another plasma factor considered as a biomarker of cardiovascular risk, namely Anti-Apo-AI antibodies. Also found in immune-based as well as inflammatory diseases, these autoantibodies are responsible for the increased risk of atherogenesis. In particular, it was discovered that these antibodies lead to an increase in the number of lipid droplets in macrophage cells. Previously, Pagano and collaborators demonstrated that this stimulus is also present in another cell type, namely the HepaRG, a cell line of hepatic origin. Therefore, in this project, carried out at the Department of Medicine of the University of Geneva under the supervision of Professor Nicolas Vuilleumier and Doctor Sabrina Pagano, we wanted to study the functioning mechanism of these Anti-Apo-AI IgG in hepatic cell model named HepaRG cells. We have discovered how these antibodies maintain unchanged the expression of a gene involved in lipid homeostasis, like SREBP-2, while stimulating the expression of another gene involved in the metabolism of triglycerides such as SREBP-1 up to 24 hours. On the other hand, other genes such as FASN and GPAT1 are down-regulated with a probable negative feedback mechanism. These results suggest that Anti-Apo-AI IgG could be a potential inducer of lipid accumulation in hepatic cells leading to fatty liver development.

We have also observed how Anti-Apo-AI IgG foster inflammation by hepatic cells and that this inflammatory response is TLR2 mediated and not influenced by SREBP-1.

To conclude through this thesis study it has been shown that Lp (a) is a lipoprotein with different implications for cardiovascular risk depending on the size, in particular, the smaller the size, the greater the concentration and CLC are, and therefore the possibility of atherogenesis.

It has been shown that CEC and CLC are excellent parameters for evaluating cardiovascular risk, thus allowing the definition of the risk in subjects suffering from pathologies even not strictly on a cardiovascular basis, such as NAFLD. In NAFLD we have discovered a possible mechanism for cardiovascular risk promotion, possessed by patients with metabolic form, which would consist in an alteration of CEC and CLC.

In this thesis we have seen how a non-pharmacological approach for risk reduction, such as the Mediterranean diet is better than the vegetarian diet, resulting in beneficial effects at CEC level.

Moreover, we have seen that Anti-apoA-1 IgG cause a strong inflammatory response and promote lipid accumulation in human hepatocytes suggesting that these autoantibodies could be a potential contributor in the development of NAFLD/NASH but these results warrants further investigations and possibly a validation in NAFLD/NASH patients.

Supplemental studies are also needed to support anti-ApoA-1 IgG as a possible link between NAFLD and CVD or other complications.

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