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Disturbances and pharmacological modulation of serum functional lipid profile in patients with cardiovascular and neurodegenerative diseases

Coordinatore:
Chiar.mo Prof. Marco Mor

Tutore:
Chiar.ma Prof.ssa Francesca Zimetti

Dottoranda: Marcella Palumbo

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Abstract

Circulating lipoproteins are currently considered to play a very important role in cardiovascular disease. In detail, High-density lipoproteins (HDL) are considered atheroprotective because their role to remove the excess of cholesterol from peripheral tissues by promoting the reverse cholesterol transport (RCT), in particular, by promoting cholesterol efflux from peripheral cells, especially from macrophage cells, which are the cells most involved in atherosclerotic plaque progression. Cholesterol efflux can occur by several pathways, including passive aqueous diffusion (AD) and active efflux, which is mainly mediated by two transporters, ATP-binding cassette G1 (ABCG1) and A1 (ABCA1). Low-density lipoproteins (LDL) are atherogenic because of their capacity to be loaded by macrophages, which may result in the formation of foam cells, a hallmark of atherosclerosis. In recent years, it has become clear that the function of lipoproteins may be more relevant to cardiovascular risk assessment than the quantification of their circulating levels. Another important factor modulating cholesterol homeostasis is Proprotein convertase subtilisin/kexin type 9 (PCSK9) which acts by degrading the hepatic low-density lipoprotein receptor (LDLR) increasing circulating LDL concentration. This underlines the importance of PCSK9 inhibitors (PCSK9-i) that by inhibiting the activity of PCSK9, reduce LDL-C levels and consequently cardiovascular (CV) risk.

These three main components, namely the HDL capacity to promote cell cholesterol efflux (HDL-CEC) and the serum lipoproteins capacity to promote cholesterol accumulation in macrophages (CLC), and the PCSK9 activity, constitute the serum functional lipid profile.

Based on these premises, the first aim of this study was to identify possible disturbances of the serum functional lipid profile in different clinical settings associated to a high cardiovascular risk, such Familial Hypercholesterolemia (FH) with Achilles Tendon Xanthoma presentation (ATX) and Abdominal aortic aneurysm (AAA), by measuring the HDL-CEC and the serum CLC. In this study, we also included the evaluation of the serum functional lipid profile in a neurodegenerative disease, the Alzheimer's Disease (AD) clinical setting, which shares many risk factors with CV, of which dyslipidaemia is the most interesting for this work. In addition, another objective of this study was to evaluate whether the possible alteration of serum functional lipid profile in these different clinical settings may be related or not to plasma levels and/or lipoprotein composition and to CV risk parameters. Secondly, the project aimed to evaluate how a pharmacological intervention in FH under PCSK9-i may have a beneficial impact on serum functional lipid profile.

Comparing FH presenting ATX and FH without ATX no significant differences were found in terms of plasma lipid profile. However, a significant increase in ABCA1-mediated HDL-CEC (+ 18.6 %) was observed in ATX compared to no ATX patients. Furthermore, in ATX-presenting patients, ABCG1-mediated HDL-CEC was lower (− 11 %) and serum CLC was higher (+ 14 %) compared to patients

without ATX. Considering all the patients together, ABCG1 HDL-CEC and serum CLC correlated with ATX thickness inversely ($p = 0.013$) and directly ($p < 0.0001$), respectively.

In AAA, ABCG1 HDL-CEC was lower (− 16 %; $p < 0.001$) and ABCA1 HDL-CEC was higher (+ 31.7 %; $p < 0.0001$) in AAA subjects compared to a control group, characterized by the same CV risk and no AAA. A stratification of the values suggests that smoking may partly contribute to these modifications. We found higher Lecithin:cholesterol acyltransferase (LCAT) (+ 23 %; $p < 0.0001$) and cholesteryl ester transfer protein (CETP) (+ 49 %; $p < 0.0001$) activity in AAA sera. HDL-CEC and CETP activity correlated with CLC only in AAA, suggesting the existence of a link between accelerated HDL function, intracellular cholesterol, and aneurysm formation.

In AD subjects compared to a cognitive normal control group, we observed normal plasma lipids levels but significantly reduced unesterified cholesterol and unesterified/total cholesterol ratio. Cholesterol efflux capacity mediated by the transporters ABCA1 and ABCG1 was reduced in AD patients' plasma, in agreement with the reduced content of small discoidal pre-β HDL in AD. LCAT activity and cholesterol esterification rate (CER), two measures of the efficiency of the esterification process, were reduced by 29 % and 16 %, respectively, in the plasma of AD patients.

In another cohort of FH patients, that underwent 6-months treatment with PCSK9-i, total cholesterol and LDL-C significantly decreased (- 41.6 %, $p < 0.0001$ and - 56.7 %, $p < 0.0001$, respectively). Despite no changes in HDL-C levels between the groups, ABCG1 HDL-CEC significantly increased after treatment (+ 22.2 %, $p < 0.0001$) as well as HDL-CEC by AD (+ 7.8 %, $p = 0.0008$). Only a trend towards reduction of ABCA1 HDL-CEC was observed after treatment. PCSK9-i significantly decreased serum CLC (- 6.6 %, $p = 0.0272$). The latter effect was only partly related to the reduction of LDL-C levels ($R^2 = 0.091$; $p = 0.006$).

In conclusion, the results observed in the three clinical settings, all potentially associated with high cardiovascular risk, generally showed a dysregulation of lipoprotein function, notably a reduced ability of HDL to mediate cholesterol efflux through the ABCG1 transporter, independently HDL-C levels, and an increased ability of serum lipoproteins to promote cholesterol accumulation in macrophage cells, independently LDL-C levels. Accordingly, in FH after pharmacological intervention with PCSK9-i, we observed an increase in ABCG1 HDL-CEC and a reduction in serum CLC, suggesting a beneficial effect of pharmacological treatment on lipoprotein function, which could contribute to the reduction in CV risk associated with this therapy. Overall, these results contribute to the identification of novel functional parameters, to be used together with the traditional one to better define the CV risk in these populations. In addition, these parameters may represent in the future new pharmacological targets for the treatment of cardiovascular and neurodegenerative diseases.

INTRODUCTION

1. Study populations

In this study we characterized the disturbances of the serum functional lipid profile and the impact of its pharmacological modulation in the following clinical settings linked to high cardiovascular risk and to neurodegenerative conditions:

-Familial Hypercholesterolemia (FH),

-Abdominal aortic aneurysm (AAA),

-Alzheimer's Disease (AD).

All these diseases, although very different from each other, share several risk factors such as an altered plasma lipid profile. Given the certain role of lipid profile levels in these diseases, an investigation was conducted in this work focusing on lipoprotein function, an emerging and clinically relevant parameter not always dependent on plasma lipoprotein levels.

1.1. Atherosclerosis and cardiovascular (CV) disease: general aspects, and risk factors

Atherosclerosis is a chronic, multifactorial, degenerative disease that affects medium-sized and large arteries and can be fatal. The World Health Organization (WHO) considers atherosclerosis-related myocardial infarction and stroke to be the leading causes of death in humans' essential structural components for cellular membrane and myelin, a precursor of steroid hormones and bile acid synthesis, but also a required component for synapse and dendrite formation[1].

The typical lesion of atherosclerosis is the atheroma or atherosclerotic plaque, which is a thickening of the intima mainly due to the accumulation of lipids, infiltration of immune cells and proliferation of connective tissue, forming a fibrous cap over the lipid core[2].

Atherosclerosis usually does not cause signs and symptoms until there is a severe narrowing or complete blockage of an artery. The disease can affect any artery in the body, including the arteries of the heart, brain, arms, legs, pelvis, and kidneys. Consequently, different diseases can develop depending on the localization of the atherosclerotic lesions[3].

The first stage of atherosclerosis is characterized by the accumulation of LDL (low-density lipoprotein) and VLDL (very low-density lipoprotein) under the endothelium, which contributes to the endothelial damage and the onset of inflammation. Atherosclerotic lesion formation is initiated by the modification of lipoproteins and the intimal infiltration of monocyte differentiated in macrophages, and subsequently by the uptake (primarily by macrophages) of these modified plasma-derived lipoproteins, resulting in foam cell formation. In addition, inadequate efferocytic removal of apoptotic cells and foam cells promotes lesion progression. This lesion can lead to complications, one of the most important of which is the rupture of the fibrous cap with the

formation of a thrombus that subsequently occludes the vessel. Thrombus-induced occlusion leads from the silent state to sudden vascular injury with acute ischemia and infarction[4] (**Figure 1**).

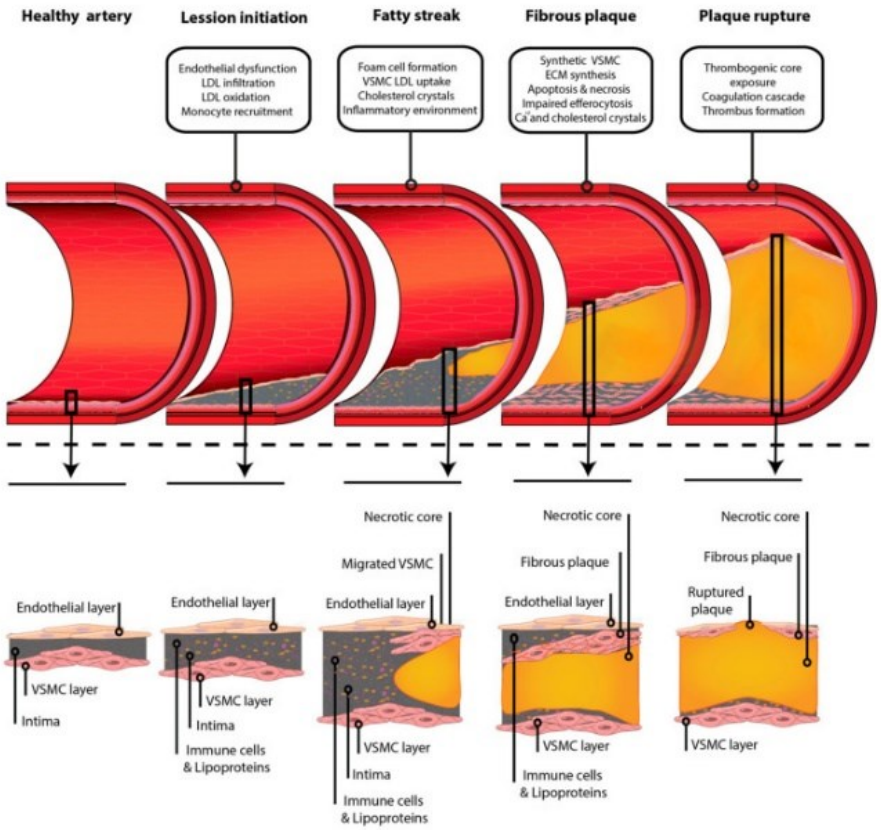

Figure 1 Schematic representation of atheroma plaque progression[5].

Atherosclerosis is a multifactorial disease, caused by the interaction of several risk factors: genetic factors, smoking, hypercholesterolemia, metabolic syndrome, diabetes mellitus, hypertension, obesity, hyperhomocysteinemia and sedentary lifestyle. Other factors include alcohol consumption, infectious agents (especially Chlamydophila Pneumoniae), certain chronic inflammatory diseases such as chronic obstructive pulmonary disease, rheumatoid arthritis, and chronic kidney disease. The plethora of possible etiologic agents and the decades-long evolution of lesions are reflected in the extreme complexity of the pathology formation process[6].

Atherosclerosis is the predominant cause of CV diseases including myocardial infarction (MI), heart failure, stroke and claudication[7].

CV diseases are one of the leading causes of premature mortality, with cardiometabolic, behavioral, environmental, and social risk factors being the major contributors to disease development. Until the 20th century, CV rates were low in low and middle-income countries, where the prevalence of infectious diseases and malnutrition was higher; however, due to the rapid economic transition in the 21st century, CV mortality rates in these countries have risen rapidly, catching up with those in industrialized nations. To date, the global prevalence of CV diseases has increased from 271 million

in 1990 to 523 million in 2019, and deaths from CV diseases have increased from 12.1 million to 18.6 million.

CV risk factors can be divided into two categories:

-non-modifiable CV risk factors; these include age, gender and genetic predisposition, among other factors.

-modifiable cardiovascular risk factors, that can be reduced or controlled by specific behaviors; examples include biological factors, smoking, physical inactivity and unhealthy diet[8].

The effect of behavioral risk in individuals with a higher risk for CV diseases, i.e. individuals with diabetes mellitus, hypertension and obesity, is particularly critical, so they have to be prevented or modified by pursuing a healthy lifestyle or through pharmacological treatments[9].

Regarding biological factors, low levels of high-density lipoprotein cholesterol (HDL-C) and high levels of LDL cholesterol (LDL-C) and other apolipoprotein B (apoB)-containing cholesterol-rich lipoproteins have been associated with an increase CV risk[10,11]. The abnormal blood lipid levels have been identified as a cause of MI, according to the INTERHEART study, and become the most important risk factor for MI[12]. The benefits of LDL-C lowering treatment is a concept increasingly supported by numerous studies, which led to the development of the slogan: "The lower the achieved LDL-C values, the lower the risk of future cardiovascular events". To date, there are not been reported adverse effects of very low LDL-C concentrations [< 1 mmol/L (40 mg/dL)][11]. LDL are also susceptible to modification by oxidizing agents; LDL oxidation and structural modification, increase endothelial activation promoting the expression of adhesion molecules and growth factors for monocytes. This also causes smooth muscle cells (SMCs) proliferation and inflammatory cell activation that leads to atherosclerotic plaque progression[13]. Also, high levels of triglycerides (TG) have been associated with an increase in CV risk[14].

For these reasons and many more, dyslipidemia is the major risk factor for CV. WHO evaluated that dyslipidemia accounted for more than 4 million deaths per year globally, responsible for 18 % of ischemic heart disease and 56 % of stroke; subjects with hypercholesterolemia and hypertriglyceridemia were found almost in one-third of the CV patients. The burden of dyslipidemia may differ among individuals and geographic areas as a function of age, disease, environment, diet, and lifestyle factors. The coexistence of lipid disorders and other CV risk factors in cardiac patients has been shown to act synergistically to the development of atherosclerosis and cardiovascular morbidity and mortality[13]. As in the general population, in autoimmune rheumatic diseases (ARDs) atherosclerosis is due to the contribution of lipid metabolism disturbances as well as inflammation[15–18]. Many abnormalities in the serum lipid profile have been described in ARDs, depending on the particular disease, but also on the medications used. Among ARDs, in Rheumatoid Arthritis (RA) subjects, we observe what could be called a "lipid paradox": in contrast to the general

population, RA patients with very low LDL levels have an increased CV risk, with a U-shaped association between the two variables[19]. The mechanisms underlying this phenomenon are still not clear, however, a large body of data has been generated in our laboratory on the potential impact of lipid alteration function on CV risk in patients with RA, as we will explore later[20–22]. These and many other works have also shown that anti-rheumatic treatment ameliorates lipid profile and function, as we will explore in detail below, and is associated with CV protection[23–25].

This also applies to the general population affected by CV diseases, for which, in order to manage these diseases and to reduce the number of people affected by these conditions, it's crucial to identify the potential contributors of dyslipidemia to discover new targets and to develop new pharmacological strategies to reduce the incidence of these disorders.

1.2. Familial Hypercholesterolemia (FH)

Familial hypercholesterolemia (FH) is a genetic autosomal dominant disorder caused by mutations in key genes involved in the catabolism of LDL. Patients may display a homozygous or a heterozygous genotype, which determines the age at which the manifestations of CV diseases will occur and the severity of the disease[26]. The syndrome is clinically characterized by extremely elevated plasma concentrations of LDL-C, which leads to a proportional increased risk of early CV diseases[27].

The diagnosis of FH is largely clinical. Several diagnostic scores have been developed for the clinical definition of FH, the most widely used of which is the Dutch Lipid Clinic Network (DLCN), which uses five main aspects of the disease: family history of hypercholesterolemia and/or early CV diseases, clinical history of early CV diseases, physical examination for evidence of pathognomonic signs, elevated LDL-C concentrations at repeated measurements, and secondarily genetic analysis[28].

Early diagnosis of FH allows the beginning of specific therapy mainly aimed at lowering cholesterol levels, combined with appropriate monitoring and possible correction of other cardiovascular risk factors.

1.2.1. Epidemiology, etiopathogenesis and treatment of FH

Evidence from two large meta-analyses published in 2020[29,30] estimates the global population prevalence of the heterozygous form of FH (HeFH) to be 1/300, which means a total of 14-34 million cases worldwide[31]. The homozygous form (HoFH) is rarer with an estimated prevalence of approximately 1/160,000 to 320,000[32].

The prevalence of FH is 18 times higher in patients with CV diseases[29] and even higher in those with early ischemic heart disease, where it is 21 times higher than in the general population[30].

However, the effective prevalence in the general FH population remains unknown and is often underestimated because it derived from hospitalized patients and disease registers and is influenced by early mortality in patients with HoFH[33].

Taken together, these data suggest that FH is vastly underdiagnosed in most countries, with less than 1% of affected individuals being diagnosed with the disease[31,34].

Familial hypercholesterolemia has a co-dominant autosomal transmission pattern. It is caused by mutations in genes encoding key proteins involved in LDL metabolism, resulting in reduced cellular LDL uptake and elevated plasma LDL-C concentrations.

In FH, mutations typically affect one of these key genes:

- 80–85% of FH cases are caused by loss-of-function mutations of the LDL receptor (LDLR) gene, responsible for the binding and internalization/catabolism of LDL, of which more than 1,600 variants have been identified to date, affecting all functional domains of the receptor protein (single nucleotide mutations, copy number variations and splicing mutations)[26,35];
- 5–10% of cases are caused by functional mutations of the APOB gene, which encode for the one apolipoprotein included in LDL, that alter the binding domain of apoB to LDLR[26];
- 2 % (approximately) of cases are caused by gain-of-function mutations of the PCSK9 gene, that binds LDLR and promotes the degradation of this receptor[26,36,37].

There is also a very rare form of FH, autosomal recessive hypercholesterolemia (ARH), caused by mutations in the LDL receptor adaptor protein 1 (LDLRAP1), a cytosolic adaptor protein which binds the cytoplasmic tail of the LDLR, mediating the endocytosis of the LDL–LDLR complex[26].

FH results from mutations in homozygosity (HoFH) or, more commonly, compound heterozygosity (different mutations in each allele of the same gene) of LDLR; rarely, there are mutations in two different loci affecting LDLR function (double heterozygotes), usually the first within the LDLR gene and the second in one of the other three loci described above[38].

It is possible to detect a pathogenic mutation in at least one of these genes in approximately 80% of subjects with "definite" FH and in 20-30 % of subjects diagnosed with "probable" FH[39,40]. Among them, 85-90 % have an inherited mutation in the LDLR gene, and the resulting clinical phenotype is referred to as autosomal dominant hypercholesterolemia (ADH-1)[41]. Two to 4 % of individuals with FH have PCSK9 mutations and 1 to 12 % have APOB mutations[41–43].

Exposure to markedly elevated plasma levels of LDL-C from birth underlies the typical clinical manifestations of FH: early atherosclerosis and development of skin and tendon xanthomas.

The concept of the cumulative burden of LDL-C[44,45] emphasizes the importance of early diagnosis and appropriate treatment[31]: when this does not occur, most patients with FH will develop their first cardiovascular events by the sixth decade of life for the HeFH form (before age 55 for men and 60 for women) and in adolescence for homozygotes[46].

Coronary artery calcifications, another marker of the disease, can be detected in heterozygotes as early as 11 to 23 years of age[47]. In addition, in the HoFH, the rapid and aggressive atherosclerotic process may involve the aortic root with impairment of the coronary valve cusps, also causing supravalvular aortic stenosis in the second decade of life[48].

In individuals with FH, exposure to elevated LDL-C over the years leads to its deposition at several sites in addition to the vascular district: at the level of the corneal margin (corneal arch), eyelids (xanthelasmas), and/or tendons (xanthomas).

In particular, xanthomas are localized lipid accumulations highly suggestive of FH, and their size is directly correlated with plasma cholesterol levels[49]. In fact, they appear in the first decade of life in individuals with HoFH, whereas in heterozygotes they usually appear after the age of 20, with an increasing prevalence with age (up to 75 % in some studies) and independent of sex. However, the distribution of xanthomas is helpful in the differential diagnosis, with FH typically occurring at the level of the Achilles tendons, the dorsum of the hands, and the extensor surfaces of the knees and elbows.

Detection of tendon xanthomas occurs primarily by objective examination through inspection and palpation of the tendon, a subjective method dependent on clinical experience and burdened by low sensitivity, which does not allow their identification in the earliest stages. In recent years, however, tendon xanthomas have been the subject of further investigation using various imaging techniques to improve the ability to identify them earlier in individuals with FH[50] (**Figure 2**).

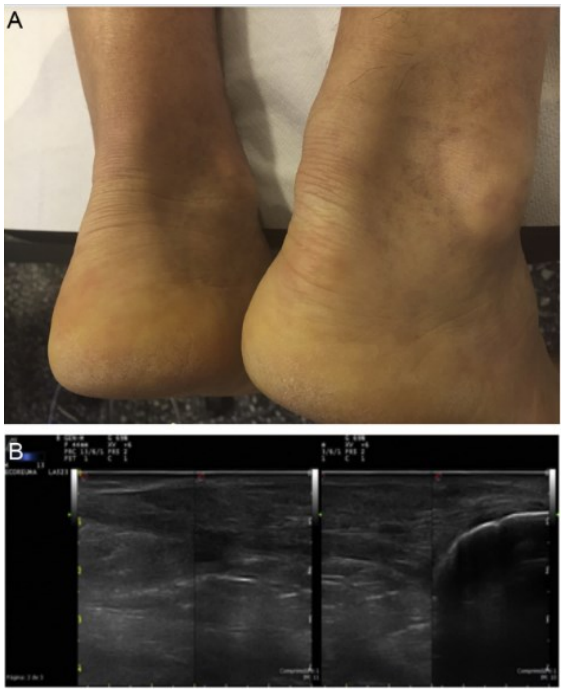

Figure 2 (A) Achilles Tendon Xanthoma. **(B)** Ultrasound of the Achilles tendon of the same patient[51].

In addition to being a pathognomonic sign of the disease, the presence of xanthomas has recently been shown to be associated with a threefold increased risk of CV diseases in patients with FH[52–56]. Specifically, in the retrospective study by Tada et al[52], xanthomas were found to be associated with coronary artery disease regardless of the presence of pathogenic mutations for FH; even in the presence of such genetic defects, there was a cumulative effect with an 11-fold increased odds of developing CV diseases ($p < 0.001$), independently of other risk factors in subjects with significantly elevated LDL-C levels, which may reflect the association of FH clinical signs with FH mutation status and other lifestyle factors or other unknown polygenic causes.

Based on the previous premises ("the greater the absolute LDL-C reduction, the greater the cardiovascular risk reduction")[11,37,57–61], and the availability of new cholesterol-lowering drugs that have achieved excellent results in terms of efficacy and safety[62,63], the recent ESC/EAS Guidelines for the Management of Dyslipidemia have proposed a further reduction in therapeutic targets[11].

Achievement of the LDL-C reduction targets recommended by the guidelines is most often possible with the therapeutic armamentarium of cholesterol-lowering drugs; seven groups of lipid-modifying drugs are currently in use in clinical practice: statins, cholesterol reabsorption inhibitors, fibrates, resins, nicotinic acid derivatives, monoclonal antibodies and small-interfering-RNA drugs[26].

Statins are the drugs of choice for the reduction of cholesterol levels and the prevention of cardiovascular disease. Statins inhibit the enzyme HMG-CoA reductase, which reduces cholesterol synthesis and lowers LDL-C by at least 50 % and TG levels by 10 % to 20 %[64]. In addition to reducing the risk of heart disease, statins promote the regression of atherosclerosis, reduce vascular inflammation, and help stabilize atherosclerotic plaques[65,66].

The combination of statins with cholesterol absorption inhibitors such as ezetimibe is considered the first-line cholesterol-lowering therapy[67]. Ezetimibe reduces LDL-C and slightly increases HDL[68].

The second common therapeutic option consists of combining statins with bile acid sequestrants, which reduce LDL-C by binding to bile acids and increasing hepatic production of new bile acids[69].

However, as soon as PCSK9 was discovered as a major actor in plasma cholesterol homeostasis[70], it was hypothesized that it represented a new target for cholesterol-lowering therapy. This awareness has paved the way for the development of several new anti-PCSK9 drug classes[71].

PCSK9 inhibitors (PCSK9-i) are a newly available class of drugs that target PCSK9, often prescribed in combination with statins for FH subjects and for the treatment of cardiovascular disease[72].

These could be monoclonal antibodies that, by binding to circulating PCSK9, prevent the formation of the complex with LDLR-apoB100 responsible for lysosomal degradation of LDLR, thus making the receptor available for recycling to the hepatic cell membrane. Currently, the commercially approved PCSK9-i are two types of humanized antibodies, Alirocumab and Evolocumab. In their respective pivotal studies[62,63], the ODISSEY and the FOURIER clinical trials, both have been shown to

significantly reduce LDL-C levels by an average of 60 %, alone and/or in combination with other hypolipidemic therapies, depending on the dose. These agents also reduce TG levels and increase HDL-C levels by 26 % and 4 %, respectively[73]. In contrast to statins, PCSK9-i also reduce plasma lipoprotein(a) (lp(a)) levels by 30-40 %[74]. In the TESLA study, the addition of Evolocumab to stable lipid therapy resulted in a 30 % reduction in LDL-C at 12 weeks in 33 HoFH subjects compared to 16 HoFH subjects under placebo. Similarly, a 36 % reduction in LDL cholesterol after 12 weeks of treatment with Alirocumab was reported in the ODYSSEY HoFH study. Both studies also showed significant reductions in other atherogenic lipids, including apoB and lp(a)[75]. Although these reductions recorded in the HoFH population, are not as pronounced as in patients with HeFH, for whom they represent the first-line of treatment, given the residual activity of the gene encoding for LDLR. Consistently, in the ARCHITECT Study (Effect of Alirocumab on Atherosclerotic Plaque Volume, Architecture and Composition), including 104 determined HeFH patients enrolled in the SAFEHEART registry (Spanish Familial Hypercholesterolemia Cohort Study), the treatment with Alirocumab in addition to high-intensity statin therapy resulted in a significant regression of the coronary plaque burden and plaque stabilization, which could explain the ODISSEY results[76]. All these results explain the success of this class of drugs in reducing cardiovascular events, as well as safety and tolerability. The siRNA Inclisiran, on the other hand, represents an alternative approach that aims to inhibit PCSK9 by blocking its synthesis in hepatocytes through inhibition of coding RNA expression. In an analysis derived from Phase III studies, Inclisiran achieved more than 50 % reduction in LDL-C in a dose-dependent manner and maintained stable levels for ≤ 6 months[77,78].

PCSK9 monoclonal antibodies have been used extensively for lipid reduction, but their use is limited by their short in vivo half-lives, resulting in frequent administration and significant costs. The future of PCSK9 inhibition could lie in the development of a new class of PCSK9-i such as vaccines, small molecule drugs, etc.[72], which may be as effective in reducing LDL-C in FH patients.

1.3. Abdominal aortic aneurysm (AAA)

Abdominal aortic aneurysm (AAA) is a localized, full-thickness dilatation of the wall of the aorta in its abdominal tract, where the aortic diameter exceeds the normal size by 50 %.

The normal diameter of the abdominal aorta is 2 cm and in 95 % of cases is less than 3 cm, varying with age, and sex. For these reasons, an abdominal aorta with a diameter tract of 3 cm or more is considered aneurysmal[79]. The AAA is generally asymptomatic until its rupture, its most severe manifestation, considered the leading cause of sudden death for elderly men[80]. AAA diameter correlates closely with rupture risk: in males this relationship is strict, while in females the aortic

scaling index (ASI) (calculated as AAA diameter (cm)/body surface area (m^2), seems to be more predictive[81].

1.3.1. Epidemiology, etiopathogenesis and treatment of AAA

AAA affects more than 700,000 people in Europe with 220,000 new cases per year. The prevalence of this disease in Italy is 2-3 % in the 65 - 70 age group, 4 % in the 71-75 age group and 4.2 % in the 76 - 80 age group[82].

AAA is characterized by an increase in diameter of about 10 % per year, with a 5-year rupture risk of 25 % for diameters of 5.0 - 5.9 cm, 35 % for diameters of 6.0 - 6.9 cm, and 75 % for diameters > 7 cm. Each year, aneurysm rupture is responsible for 6,000 deaths in Italy. Approximately 80 % of patients die before access to surgery, with an emergency mortality rate of 50 % versus 5 % for elective surgery[83].

Epidemiological data indicate that the main risk factors for the occurrence of AAA are older age, the Caucasian race, a positive family history, male sex, and smoking. The lower prevalence of the disease in female subjects has been attributed to hormonal influences, as for example, the protective effect of estrogen[84]. Additionally, risk factors of AAA rupture, in addition to diameter, are growth velocity, smoking, and hypertension[85].

Smoking is recognized as the main modifiable risk factor for the development of AAA, especially when associated with male sex, as confirmed by a series of meta-analyses conducted in 2018, with ORs of 5.93 and 2.97 for the two factors, respectively. Indeed, in countries where cigarette consumption is lower, a lower prevalence of AAA is observed[86]. Smoking correlates closely with the diameter of AAA, which is eight times higher in smokers than in nonsmokers[87]. Consistently, AAA risk is reduced in former smokers. For example, In the Multicentre Aneurysm Screening Study (MASS), the benefits of smoking cessation were shown to result in decreased cases of aortic rupture[88].

The wall of the healthy abdominal aorta consists of three layers: the *tunica intima*, the innermost layer; the *tunica media*, the middle layer; and the *adventitia*, which is the outermost layer. All together, these layers form a uniform wall with a thickness of about 0.2 cm. The *intima* is characterized by a single layer of endothelial cells supported by a subendothelial layer of loose connective tissue. This is separated from the *media* by an inner elastic lamina composed of elastin fenestrated fibers. In contrast, the *media* consists of a series of 28 to 30 concentric layers of SMCs arranged in a helical shape separated by layers of reticularly arranged elastin fibers, proteoglycans, and glycoproteins. The *adventitia* is mainly composed of type I collagen fibers and a smaller number

of elastin fibers and sporadic fibroblasts. Generally, a healthy wall is composed of 45.5 % collagen (60 % type I and 22 % type III), 30.1 % elastin, and 22 % SMCs[83].

The aortic wall in AAA shows structural disorganization, with loss of smooth muscle fibrocells and increased collagen content. Fibroblasts and red blood cells, granulocytes and other inflammatory cells are found in the *media* and *adventitia*. In addition, many tissue samples manifest intramural voids lined with large amounts of non-constitutive cell types, most commonly inflammatory cells. The AAA wall also appears hypervascularized as a result of hypoxic phenomena. The percentage composition of wall proteins varies widely among subjects, with reported values of elastin between 18 % and 80 %, and collagen between 28 % and 66 %. The percentage of SMCs reported varies between 21 % and 61 %. The concept of impaired elastogenesis in AAA is commonly accepted, but with regards to changes in collagen content, the literature data are not consistent, reporting increased, decreased, or no changes in the interstitial matrix and collagen levels[83]. In the AAA wall, the expression and activity of metalloproteinases (MMPs), enzymes responsible for the degradation of collagen and elastin fibers, increases. The most important are MMP-1, MMP-2 and MMP-3, which are produced by resident cells of the wall, and MMP-9, which is mainly synthesized by cells of the inflammatory response[83].

Interestingly, inflammation is considered a central player in the development of AAA[89]. Proteases secreted by inflammatory cells can induce degradation of the extracellular matrix (ECM). Concurrently, as a result of ECM structural damage and decreased resistance of the tunica media, soluble blood components such as inflammatory cells are transported and accumulate within the tunica media via the extensively vascularized adventitia, leading to the infiltration of inflammatory cells into the vascular media. These processes, together with thrombocyte accumulation and coagulopathy activation, lead to intraluminal thrombosis and subsequent aortic dilatation and increased susceptibility to AAA rupture[90]. Intraluminal thrombosis reduces the resistance of the aortic wall by creating an inflammatory microenvironment containing neutrophils, cytokines, proteases, and reactive oxygen species. These phenomena suggest that inflammatory cells play a central role in the whole process[89].

During the process of aneurysm progression, the *media* thins out and the *adventitia* thickens, while the total wall thickness remains constant, in agreement with the hypothesis of active wall remodeling[83].

Changes in relative composition of wall tunics but especially profound structural alterations in the interstitium and cellular component underline the progression of the disease and its complications. A schematic histopathological description of the changes is described in **Figure 3**.

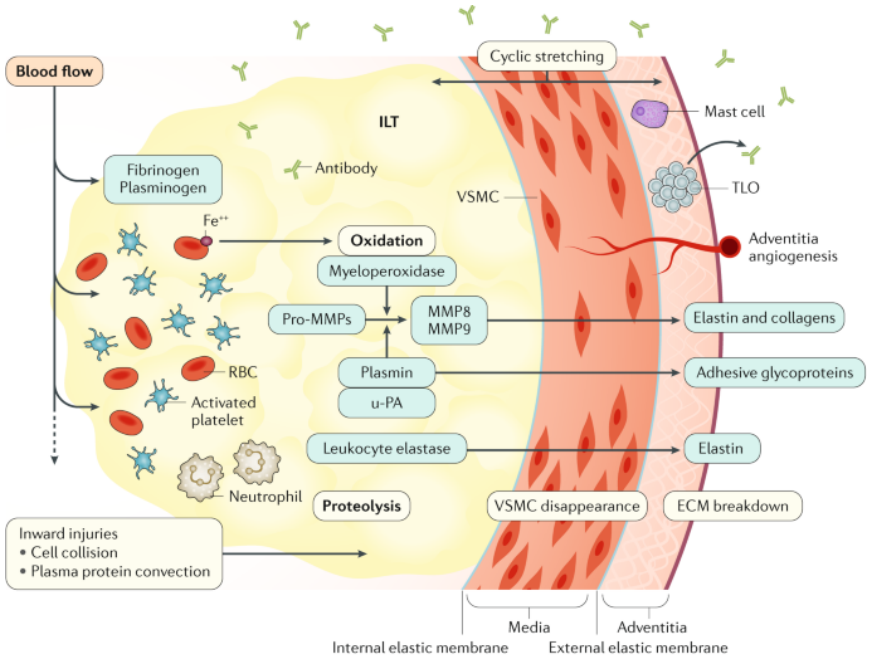

Figure 3 Representation of histopathological changes in AAA[91].

The role of a genetic component in the etiopathogenesis of AAA is recognized. Genetic studies, in addition to determining the constitutive component of the disease, are useful to identify the genes associated with AAA; to this aim genome-wide association studies (GWAS), have been used. Thanks to these, approximately 90 AAA-associated genes have been identified over the past 20 years. The mutations identified are involved in some of the most important cellular mechanisms, namely: extracellular matrix protein degradation, encoding immune factors, cell cycle regulation in vessel SMCs, lipid metabolism, and the renin-angiotensin system[84].

The immune system also plays a role in aneurysm development. In this regard, previous studies have suggested that the development of AAA is driven, partly, by Th2 helper lymphocytes. Interleukin (IL)-6, IL-8 and that pro-inflammatory cascade factors are overexpressed in aneurysm, confirming the inflammatory involvement in the pathogenesis of this disease[84].

AAA is asymptomatic in most cases and becomes visible mainly in relation to its rupture, its worst complication; the identification of novel screening strategies for early diagnosis is thus very important. Indeed, knowing about the presence of AAA allows to monitor its evolution, to target intervention on modifiable risk factors, and to plan elective surgical approach. Ultrasonography is the screening method of choice for AAA, it is inexpensive, accurate, safe, fast, and noninvasive[88]. Generally, the screenings are annual and get intensified if the rate of aneurysm growth increases; the periodicity at which the subject should be screened is determined case-by-case[84]. After the

initial diagnosis of AAA, there are three main options for the clinician to proceed: keep on monitoring the size of the aneurysm, endovascular repair, or "open" transabdominal repair; the decision of which treatment to follow up with depends on the size of the aneurysm, which is closely related to the risk of rupture. Surgery is not taken into account for diameter values less than 5.5 cm, otherwise the risk of dying from the surgery is greater than the risk of aneurysm rupture[84]. Endovascular repair has the advantage of reducing perioperative morbidity, but it requires follow-up and the risk of a second surgery is high; an "open" repair, on the other hand, is associated with higher perioperative risk but provides better long-term outcomes, with lower need for follow-up or second surgery[84].

Because there are no effective treatments that can be administered at a relatively early stage, there are no indications that AAA can be treated at a later stage. Except for the reduction of modifiable risk factors with proper lifestyle, no other treatments, e.g., pharmacological, acting on aortic wall pathology are available to date; for asymptomatic patients and smaller aneurysms, a therapy for cardiovascular risk reduction is suggested, in particular, a nicotine replacement therapy or a smoking cessation program are recommended, and of course the use of statin[92,93]. This is mainly because the cellular and biochemical mechanisms involved in aortic tissue damage are not sufficiently known[84]. Results of studies in animal models, which indicated doxycycline as effective in blocking AAA growth through inhibition of MMP-9, have not been confirmed in humans. Another potential treatment examined, with the antihypertensive angiotensin-converting enzyme (ACE) inhibitors, is not unequivocally effective. Some meta-analyses seem to confirm the slowing of aneurysm growth in patients with AAA on statin therapy[94], and a Danish retrospective study indicates that statin use is associated with reduced risk of AAA rupture[95]. Numerous trials have been conducted to evaluate the effect on plaque reduction of novel hypolipidemic therapies (such as PCSK9-i), antihypertensive drugs, mast cell stabilizers, an antiplatelet drug, or fenofibrate, but none have shown convincing results, emphasizing the need for additional studies [94].

1.4. Alzheimer's disease (AD)

Alzheimer's disease (AD), the most prevalent cause of dementia, is a chronic neurodegenerative disorder that leads to the progressive deterioration and loss of cognitive function; AD evolves from a preclinical phase to mild cognitive and/or behavioral impairment and then Alzheimer's disease dementia. Because the disease is characterized by this wide range of cognitive symptoms of different severity, the progression of AD can be assessed using the Mini-Mental State Examination (MMSE). This test consists of a series of questions, which places the patient into categories based

on the impairment of brain activity: severe impairment (score < 18), mild or moderate impairment (18 - 25), normal cognitive activity (26 - 30)[96].

AD is characterized by two main pathological hallmarks: the progressive accumulation of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs)[97,98]. To date, AD is the fifth main cause of worldwide death among people 65 years aged and older, as well as the most common form of dementia: about 60-70 % of dementias are attributable to AD[97].

The prevalence of AD doubles from age 65, while the incidence of the disease increases exponentially until age 85, especially in women. In the female population, in fact, the prevalence of the disease is increasing: it is estimated that the prevalence of AD in women is 1.7 larger than in men, probably because their average age of life is higher, compared with men[99].

AD is difficult to diagnose, as the earliest stage comprises a long asymptomatic phase, and as some of its symptoms may be attributable to other conditions, such as Parkinson's disease, stroke, or non-AD dementia, leading to late or misdiagnosis, so the actual incidence and prevalence of the disease may be higher than estimated[98,100].

1.4.1. Epidemiology, etiopathogenesis and treatment of AD

Worldwide, the number of people with dementia is expected to triple by 2050 due to an aging population, increasing the risk of disability, disease burden and healthcare costs[99].

In Europe, the prevalence of AD is 4.4 % in the age group of 60 - 65 years, in women it represents 0.7 % between 65 - 70 years to 23.6 % over 90 years; in men, however, the values are lower: 0.6 % between 65 - 70 and 17.6 % for those over 90. The incidence of the disease in Europe is 0.9 cases per 1,000 person-years in males between the ages of 65-70 and 20 cases for the 90s; in women it starts at a rate of 2.2 cases between the ages of 65 - 70 to 69.7 cases for those over 90[101].

Italy has one of the oldest populations in the world, with more than one million people estimated to suffer from dementia[102]. According to the WHO data report published in 2020, dementia deaths in Italy represented the 7.64 % of total deaths. In recent years, the incidence of AD in Italy has shown an increasing average trend, affecting about 600,000 people, which can be interpreted by population aging and consequent increase in dementia[103].

As stated above, aggregation of Aβ is one of the most involved factors in the etiopathogenesis of the disease: improper folding of Aβ results in the formation of extracellular deposits of different sizes of this protein, leading to loss of neuronal function (**Figure 4**). The deposition of fibrillar formations of Aβ begins in the neocortex, expanding to the entorhinal region, hippocampus, subcortical area, brainstem, and eventually reaching the cerebellum. These aggregates have been termed "senile plaques" and first identified in the 900s on brain sections from subjects with dementia; their composition based on β-amyloid protein was then defined[104,105].

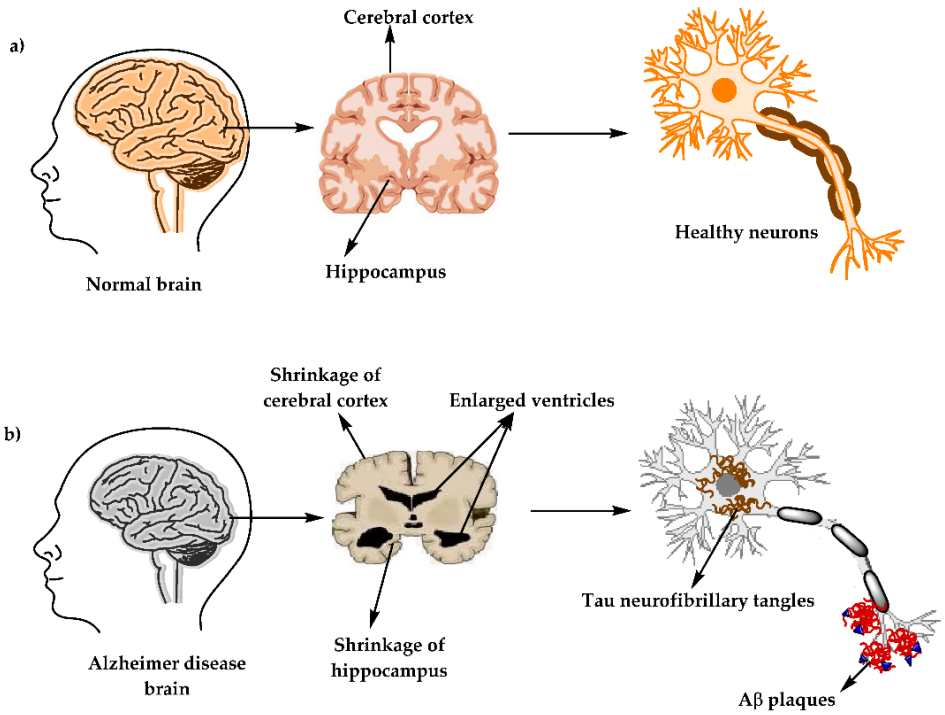

Figure 4 (a) Healthy brain and **(b)** Alzheimer's disease (AD) brain physiological structure[106].

Aβ is derived from a precursor, namely the β-amyloid precursor protein (APP), and encoded by the APP gene, located on chromosome 21. This precursor is synthesized at the endoplasmic reticulum (RE) and then transported by the Golgi apparatus to the trans-Golgi network (TGN) where it is stored[107].

APP protein is found expressed at the brain level on astrocytes, but also on endothelial cells, smooth muscle, and peripheral cells; it is processed by α, β, γ secretase enzymes having the task of cleaving the protein at different sites. In case there is a mutation on APP, the β-secretase enzyme 1 (BACE-1) processes the N-terminal portion of APP to generate the APPsβ fragment; cleavage of the C-terminal end results in the β-CTF fragment (C99), which is subsequently cleaved by γ-secretase to from insoluble Aβ protein monomers, responsible for the formation of β-amyloid plaques[108].

Aβ is a 39 - 42 amino acid, 4 kDa peptide with β-sheet conformation, which can have different isoforms; among the various isoforms, those having a length of 40 - 42 amino acids, $A\beta_{40}$ and $A\beta_{42}$,

are the most pathogenic forms. $A\beta_{40}$ constitutes 90 % of the β-amyloid plaque deposition in the brain, while the latter is the predominant form during the early stages of AD; $A\beta_{42}$ is also the most neurotoxic isoform[104,109].

Tau protein (τ) also turns out to be involved in the etiopathogenesis of AD; the τ protein is encoded by the Microtubule Associated Protein Tau (MAPT) gene on chromosome 17 and is a member of the microtubule-associated protein (MAP) family, which is abundantly expressed in the brain. In the brain, τ protein exists as a phosphorylated and soluble form, in six isoforms, which differ in their ability to stabilize microtubules and in the number of binding domains. In case of incorrect or excessive phosphorylation, τ becomes insoluble and folds back on itself resulting in polymerization into paired helical filaments (PHFs), which are responsible for the formation of neurofibrillary tangles (NTFs), an additional etiopathogenetic feature of AD[110]. The accumulation of neurofibrillary tangles begins in the entorhinal cortex and then proceeds to the limbic system, hippocampus and associative cortex, eventually invading most brain areas[111].

Magnetic resonance imaging (MRI) can be performed to show atrophy of the hippocampal area, caused by the deposition of Aβ and τ proteins[96].

Numerous evidence has documented the coexistence of different risk factors that may increase the development of AD. As for CV, also risk factors for AD can be divided into non-modifiable and modifiable; among the non-modifiable ones we find age and sex: in women it has been shown that the incidence and prevalence rate of the disease is higher than in men[99]. Also, genetics is an important non-modifiable risk factor: mutation of the APP, pre-senilin 1 (PSEN1) and pre-senilin 2 (PSEN2) genes affect the chance of developing AD, particularly, in the familial form of the disease, by resulting in excessive production of the Aβ protein[112]. Among the mutated genes that may lead to an increased risk of disease onset, apoE definitely emerges and more specifically the ε4 allele of the apolipoprotein E gene (APOE4), as we will discuss below[113].

Modifiable factors include social risk factors such as low level of education, stress and depression, insomnia or sleep disorders, sedentary lifestyle, malnutrition, alcohol abuse, smoking addiction, cardiovascular disease and hypercholesterolemia. In the pathogenesis of CV diseases and AD, aging-related cellular and molecular processes such as low-grade inflammation are the major players. Because the incidence of dementia and CV are independent, this suggests the presence of overlapping molecular mechanisms[114]. For example, serum lipid level alteration might play an important role also in the process of AD. Specifically, hypercholesterolemia, the increased plasma LDL content, leads to an increased Aβ deposition and altered blood brain barrier (BBB) permeability[99].

Currently, there are only two classes of approved drugs to treat AD, including inhibitors of cholinesterase enzyme (AChEIs) and antagonists of N-methyl d-aspartate (NMDA), which are effective only in treating the symptoms of AD, but do not cure or prevent the disease.

There are three AChEI drugs approved for the treatment of the disease in individuals with mild to moderate cognitive impairment: donepezil, rivastigmine, and galantamine.

1) Donepezil, administered orally or transdermal at a dosage of 5 -10 mg per day, is a reversible inhibitor of the enzyme AChE having the function of increasing acetylcholine concentration and consequently synaptic transmission mediated by that neurotransmitter[106,115].
2) Rivastigmine is an inhibitor of AChE and BuChE. Its activity leds to brain acetylcholine deficiency with advancing age, so an attempt was made to design a drug that had dual action, inhibiting both enzymes. It is administered either orally, as a tablet, or as a transdermal patch, and in this case, it is also indicated for the treatment of Parkinson's disease[116].
3) Galantamine is the third reversible AChE enzyme inhibitor drug approved by the Food and Drug Administration (FDA); administration of 24 - 32 mg capsules resulted in improved prognosis and in increased cognitive abilities[117].

Memantine is a glutamic acid NMDA receptor antagonist, approved by the FDA in 2003; it is a noncompetitive NMDA receptor antagonist employed to treat moderate to severe forms of the disease. As already mentioned, glutamate is the main excitatory neurotransmitter in the central nervous system (CNS), and if present in excess, its binding to the NMDA receptor results in intracellular calcium accumulation, which is responsible for neurodegenerative processes. Memantine, by binding with the NMDA receptor, prevents the NMDA receptor from binding glutamate, exerting a neuroprotective action resulting in improved ability to articulate speech and reduced chance of developing complications such as irritability, delusion, and hallucinations[118].

The use of statins for the treatment of hypercholesterolemia, has shown as an off-target effect a reduction in Aβ and τ protein levels, particularly lovastatin and simvastatin[114]. Lovastatin and other lipophilic statins have led, in different *in vivo* studies, to the improvement of NMDA glutamatergic receptor function, ChAT enzyme and exert neuroprotective action on neuronal loss by reducing the levels of the cytokines IL-6, TNF-α and IL-1β[119]. The role of statins in AD is still controversial, as recent meta-analyses have shown overall a neutral effect of statins, to recommend this treatment for the prevention of dementia[120].

After years of failed attempts to develop a disease-modifying therapy for AD, consistent evidence in support of clinical efficacy was finally presented for a monoclonal antibody targeting the Aβ protofibrils[121]. Aducanumab, a human mAb, is the first FDA approved drug for AD since 2003, and the first drug that specifically targets Aβ aggregates (insoluble fibrils and soluble oligomers).

Aducanumab has shown promise in reducing brain Aβ content and slowing cognitive decline in clinical trials. However, controversies surround its approval due to contradictory study results, high costs, and reported adverse events. Aducanumab seems effective primarily in mild or early AD stages but future research with a larger population over an extended period is needed to assess its overall efficacy and safety for AD treatment[122]. Even Lecanemab (BAN2401), derived from a murine mAb158, targets Aβ protofibrils. In a clinical trial involving 1795 early AD patients, it significantly slowed disease progression over 18 months compared to placebo. The drug demonstrated superiority in terms of quality of life and caregiver burden. In addition, biomarkers indicated favorable changes in tau pathology and neurodegeneration, confirming its potential disease-modifying effects. Based on these promising results, the FDA granted accelerated approval to Lecanemab in January 2023, making it the second drug ever approved for the treatment of AD[121].

2. Definition of Serum functional lipid profile

Serum functional lipid profile takes into consideration three main factors that synergistically are involved in the regulation of the cellular cholesterol homeostasis, the alteration of which we observe the formation of foam cells, the main cells involved in the atherosclerotic process.

Evidence indicates that a correlation exists between serum functional lipid profile and cardiovascular risk, independently of classical risk factors such as high-density and low-density lipoproteins cholesterol (HDL-C, LDL-C) representing the quantitative lipid profile. For this reason, we believe it is more accurate to talk about serum functional lipid profile in the context of cardiovascular risk assessment.

2.1. Macrophages cholesterol homeostasis regulation

Overall, macrophage cholesterol homeostasis depends on the balance among several processes, including cellular cholesterol synthesis, efflux, uptake, and PCSK9 activity.

Cholesterol is a substance of fundamental importance in humans as it's a component of eukaryotic membranes and an important precursor of steroid hormones and bile acids. The ratio of free cholesterol (FC) and phospholipids in cell membranes has been shown to be important in maintaining the proper fluidity of the membrane itself[123]. The biosynthesis of cholesterol starts with acetyl coenzyme A (acetyl-CoA) and involves several enzymatic reactions. Among these, the reduction step of coenzyme A 3-hydroxy-3-methylglutaryl (HMG-CoA) to mevalonate catalyzed by HMG-CoA reductase (HMGCR) is the limiting step, suggesting that regulation of HMGCR is crucial for cholesterol biosynthesis[124].

Cholesterol biosynthesis is a biologically highly regulated process[125]. Sterol regulatory element binding proteins (SREBP) binds to the sterol regulatory element (SRE) in the proximal promoter region of HMGCR and activates its transcription to accelerate cholesterol biosynthesis[126]. SREBP also binds SRE in the promoter of the LDLR, the molecule responsible for the clearance of LDL-C in the liver[126]. When the cellular level of cholesterol is reduced, mature SREBP is increased by sphingosine 1-phosphate protease (S1P) and, as a result, HMGCR expression is activated. Conversely, an increase in cellular cholesterol levels inhibits HMGCR expression[127].

The excess of FC cannot be catabolized by most peripheral cells and tissues, so cholesterol efflux to extracellular HDL could protect cells against FC accumulation; cholesterol homeostasis mainly depends on macrophage (the main cell involved in atherosclerotic plaque progression) cholesterol content and protein composition and size of HDL, which act as cholesterol acceptors efflux, through various transporters[128].

As previously described, cholesterol accumulation in macrophage cells can lead to the formation of atherosclerotic plaques and the formation of cholesterol crystals, a hallmark of advanced plaques[129–131]. Cholesterol crystals can stimulate the formation of the NOD-, LRR-, and pyrin-protein 3 (NLRP3)-containing inflammasome and promote inflammation, which accelerates atherogenesis, induces arterial inflammation, and contributes to the destabilization of atherosclerotic plaques[132–134].

In 2003, Nabil Seidah and coworkers discovered PCSK9[70]. PCSK9 is synthesized in the liver and then secreted into the plasma. Circulating PCSK9 can bind hepatic LDLR and avoid receptors' recycling[135,136]. Reduced cell surface LDLR leads to defective LDL-C excretion and elevated LDL-C levels, which has prompted a massive technological effort to develop innovative pharmacological strategies aimed at inhibiting PCSK9[137,138]. PCSK9 is also responsible for several extrahepatic effects. For instance, in macrophages, PCSK9 interferes with cholesterol efflux, by directly inhibiting ABCA1-mediated pathway through the downregulation of ABCA1 gene and protein expression, and by increasing macrophage cholesterol content by increasing scavenger receptor expression, effects that promote foam cell formation[139,140].

All pathways of cholesterol homeostasis could be regulated, leading to advances in the development of therapeutic strategies. The feedback from clinical observations has further advanced the study of cholesterol homeostasis, promoting clinical progress.[141].

Cholesterol homeostasis is involved in the development of several diseases and is determined by the processes of biosynthesis, absorption, efflux, transport, storage, utilization and/or excretion. Therefore, in this chapter, we will summarize the main processes involved in the regulation of cholesterol homeostasis by serum HDL and LDL lipoproteins and PCSK9 and the main strategies for their modulation.

2.2. HDL cholesterol efflux capacity

2.2.1. HDL heterogeneity

HDLs are a heterogeneous class of lipoproteins composed of several subpopulations that differ in protein content and morphology mainly formed in the gut and liver (**Figure 5**). HDL is the smallest lipoprotein particle with a mean size of 8–10 nm and density of 1.063–1.21 g/ml[142], mainly in spherical shape having a neutral lipid core, composed of small variable amounts of cholesterol ester (CE) and triglycerides and abundant amounts of phospholipids and proteins; the most common apolipoprotein in HDL are apoAI and AII, which account for 70 and 20 % respectively of the proteins present in HDL, and other many apolipoproteins as apoAIV, apoC, apoD, apoJ, apoM and E, to a lesser degree[143,144]. HDL can be divided into two main classes based on apolipoprotein content and in particular: LpAI, which contains only apoAI, and LpAI:LpAII, which contains both apoAI and apoAII[145]. LpAI particles can be further divided into subclasses based on the number of apoAI molecules to which they are bound, with a progressive increase in size. LpAI:LpAII particles always contain two apoAI molecules and a variable number of apoAII, up to 3[146].

As a result of the action of various plasma and cellular factors, HDL undergoes continuous remodeling processes that lead to the formation of several subpopulations. Based on density, HDL could be divided into small and dense HDL called HDL3 (1.125–1.21 g/mL), further divided into HDL3a, HDL3b and HDL3c, and big ones called HDL2 (1.063–1.125 g/mL), also divided into HDL2a and HDL2b. Two-dimensional agarose gel electrophoresis can be used to detect the migration of mature α-HDL, with high negative charge and the most represented circulating HDL, and pre-β HDL, consisting of nascent discoidal, poorly lipidated HDL mainly contain apoAI and phospholipids[147]. Usually, discoidal pre-β HDL are transformed by the activity of the enzyme LCAT into mature, spherical particles, α-HDL[147]. LCAT is a 63 kDa glycoprotein synthesized in the liver and is the sole enzyme capable of esterifying cholesterol in plasma; it catalyzes the transfer of two acyl groups from lectin to free cholesterol, forming cholesterol ester and lysolectin. The cholesteryl ester migrates within the lipid core, primarily due to its hydrophobic nature, contributing to the formation of mature HDL[148]. LCAT activity plays a critical role in the metabolism of HDL, and a genetic deficiency of this enzyme in humans is associated with a marked decrease in HDL cholesterol and apoAI levels[149]. Also, a mutation in LCAT yields a reduction in the content of HDL2 and an accumulation of pre-β HDL[150]. The activity of this enzyme is fundamental in promoting the Reverse Cholesterol Transport (RCT) process (see below), because it is able to maintain a constant HDL concentration gradient between plasma and cells.

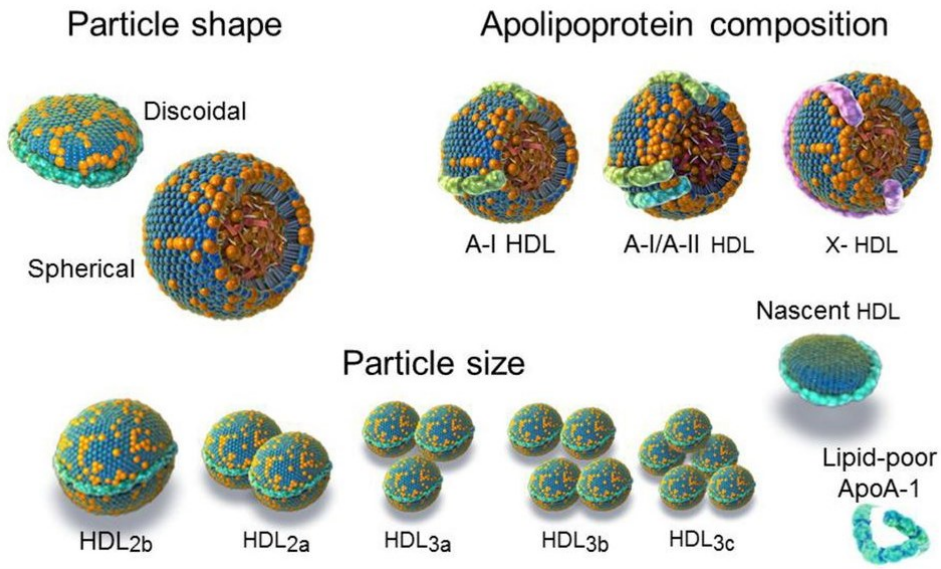

Figure 5 Heterogeneity of high-density lipoproteins (HDL)[151].

2.2.2. Reverse Cholesterol Transport (RCT)

HDL are involved in many atheroprotective properties: they are anti-inflammatory, they have antiapoptotic and antioxidative activity; they prevent LDL oxidation, a fundamental step in the formation of foam cells and atheroma[152]; and they have vasodilatory and antithrombotic activity[153]. Among this multitude of protective activities, the most studied function of HDL is the capacity to promote the transport of peripheral cholesterol to the liver, to be then excreted in bile and finally in feces, a process called RCT[154]. The transport of peripheral cholesterol to the liver is necessary to balance the uptake and de novo synthesis to maintain constant cholesterol homeostasis throughout the body[155]. According to the hypothesis of Glomset and Ross, there is an inverse relationship between RCT and atherosclerosis, as atheromatous formations appear when there is an imbalance between the accumulation and the removal of cholesterol in the arteries[154,156]. Few years later, based on an inverse relationship between HDL cholesterol and cardiovascular disease, it was proposed that increased levels of HDL should lead to a relative increase in the clearance of cholesterol from the artery wall, with consequent benefits in the prevention of related diseases[157]. Recently, several studies have been focused on the concept of "macrophage RCT" because macrophages are the most involved cells in atherosclerotic lesions.

The mechanism of RCT could be divided into these fundamental steps:

- cellular cholesterol efflux from macrophages to extracellular cholesterol acceptors (HDL) via specific cholesterol transporters (this is the first limiting step of the process);
- HDL remodeling in plasma;

- cholesterol uptake from hepatic cells and excretion via the faeces[128] (**Figure 6**).

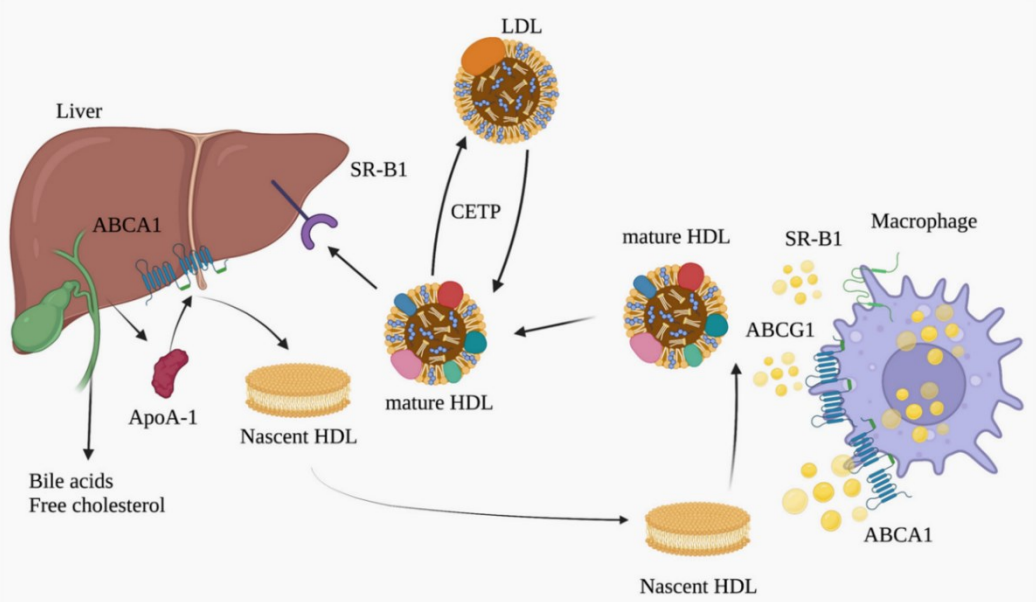

Figure 6 Schematic illustration of reverse cholesterol transport (RCT)[158].

2.2.3. Mechanism of cholesterol efflux

In the first step of RCT, excess cholesterol is transferred from arterial macrophages to extracellular HDL[159]. Excess of free cholesterol is toxic to cells, and this can be a very important factor considering that most cells in peripheral tissues (except steroidogenic organs) cannot catabolize cholesterol. Therefore, macrophages protect themselves from the accumulation of free cholesterol by converting it into CE for intracellular storage or by promoting its efflux to extracellular acceptors such as HDL[160].

Cellular cholesterol may be accepted by different subclasses of HDL, that specifically promote cholesterol efflux through different pathways:

- *Aqueous diffusion:* cholesterol can diffuse through the aqueous phase between cell membranes and liposomes. The aqueous diffusion (AD) mechanism involves a simple diffusion of cholesterol, driven by the concentration gradient, and depends on acceptor size, as extracellular mature HDL[128].
- *SR-BI:* Scavenger receptor class B type I (SR-BI) is a member of the CD36 scavenger receptor superfamily. It is a facilitated passive diffusion transporter which promotes a bidirectional flow of free cholesterol between cells and mature HDL particles across a cholesterol concentration gradient[128]. The current model of action of SR-BI suggests the transfer of cholesterol ester between HDL and the plasma membrane through the hydrophobic channel along the concentration gradient[161].

- *ABCA1:* ATP-binding cassette (ABC) transporters are transmembrane transporters involved in the transport of a wide variety of substances across endo- and extracellular membranes using the energy of ATP hydrolysis[162]. The structure of ABCA1 consists of two hydrophobic transmembrane domains, each of which contains six α-helices, and two hydrophilic domains[163]. ABCA1 is a protein that plays an important role in HDL biosynthesis and cellular cholesterol homeostasis as mutations in ABCA1 can cause an autosomal recessive disorder called Tangier Disease, characterized by low plasma HDL levels, cholesterol deposition in tissue macrophages, and prevalent atherosclerosis[164]. ABCA1 mediates the release of phospholipids and free cholesterol from the plasma membrane of macrophages to apoAI and discoidal HDL in the extracellular phase, generating lipid-poor apoAI, with one apolipoprotein molecule, and nascent discoidal HDL particles containing two, three, or four apoAI molecules per particle. Nascent HDLs are heterogeneous in size and composition and contain the major classes of lipids found in the plasma membrane[165]. The ability of ABCA1 and other related ABC transporters to promote cellular efflux and enhance reverse transport of cholesterol from peripheral macrophages to the liver underlies their cardioprotective properties[164].
- *ABCG1:* ABCG1, as ABCA1, is an ATP-binding cassette transporter involved in cholesterol efflux in macrophages. It transports cholesterol from the inside to extracellular acceptor, the mature HDL, phospholipid- and cholesterol-rich. Structurally, ABCG1 contains a hydrophobic transmembrane domain of six α-helices and a hydrophilic nucleotide-binding domain. Because ABCG1 is an emi-transporter, it dimerizes to carry out its function. Expression of ABCG1 results in an increased rate of cholesterol desorption and increased cholesterol available for both efflux and esterification[166]. Absence of ABCG1 may reduce HDL-dependent cholesterol removal and increase inflammatory signaling leading to massive lipid accumulation. Genetic polymorphisms in ABCA1 or ABCG1 correlate with the risk of developing atherosclerosis[167].

2.2.4. Cholesterol efflux capacity of HDL (HDL-CEC) and CV risk

The capacity of HDL in serum to accept and transport cholesterol through different cellular efflux pathways, also known as HDL cholesterol efflux capacity (HDL-CEC) can be measured by validated methods[160]. There are various methods *in vitro* for this evaluation: the best-known *in vitro* techniques use for example fluorescent tracer, or the BODIPY assay, using a stain for neutral lipids as a tracer for nonpolar lipids. However, the radioactive assay is considered the "gold standard"[168], as we will discuss in detail below.

Epidemiological studies define HDL as the most relevant plasma factor with atheroprotective activity in humans: 1 mg/dL increase in plasma HDL-C corresponds to a 3 – 4 % reduction in mortality from cardiovascular events[169,170]. However, induced increases of HDL levels both genetically and pharmacologically have failed to demonstrate clear benefits for cardiovascular outcomes[171,172]; also, multiple findings support the premise that alterations in HDL-C levels do not consistently impact CV risk [173]. Consequently, the importance of HDL functionality rather than quantity, emerged as pivotal in CV risk assessment, compared to HDL-C levels quantification alone[128,174].

The hypothesis of HDL function takes into account HDL quality and structural composition. Individuals with CV not only exhibit low HDL levels but also substantial alterations in their composition, and consequently, in their function[175,176].

As said before, the capacity of HDL to promote cholesterol efflux from macrophages (HDL-CEC) could be measured with an in vitro standardized, radio-based technique that allows to distinguish among the various mechanisms of efflux through the different pathways involved[177].

The relationship between HDL-CEC and CV has been extensively studied[178]. In particular, results from a recent meta-analysis showed that HDL-CEC inversely relates to CV risk, independently of HDL-C levels[179]. In two distinct subject cohorts, HDL-CEC displayed a robust, inverse association with both carotid intima–media thickness, a marker of subclinical atherosclerosis, and the likelihood of angiographic coronary artery disease[180]. This observation was further validated in a healthy subject population where ABCA1-mediated HDL-CEC inversely correlated with pulse wave velocity (PWV), an indicator of arterial stiffness and sub-clinical atherosclerosis, also in this case independent of HDL-C serum levels[181]. Additionally, the role of HDL-CEC as a marker of cardiovascular protection was strongly supported by a prospective study demonstrating HDL-CEC's robust predictive power for incident cardiovascular events in a population-based cohort without cardiovascular disease at the beginning, followed over a 9-years period[181]. In addition, in a cohort of RA subjects analyzed in our laboratory, it was observed that ABCG1 HDL-CEC inversely associated with plaque burden and vulnerability, and plaque progression (**Figure 7**)[22].

Overall, this evidence leads to the conclusion that HDL-CEC may potentially become a valuable biomarker of CV risk in the future[173].

Enhancing HDL function, particularly in terms of HDL-CEC, might improve atheroprotection and prevention strategies, as well as pharmacological treatment.

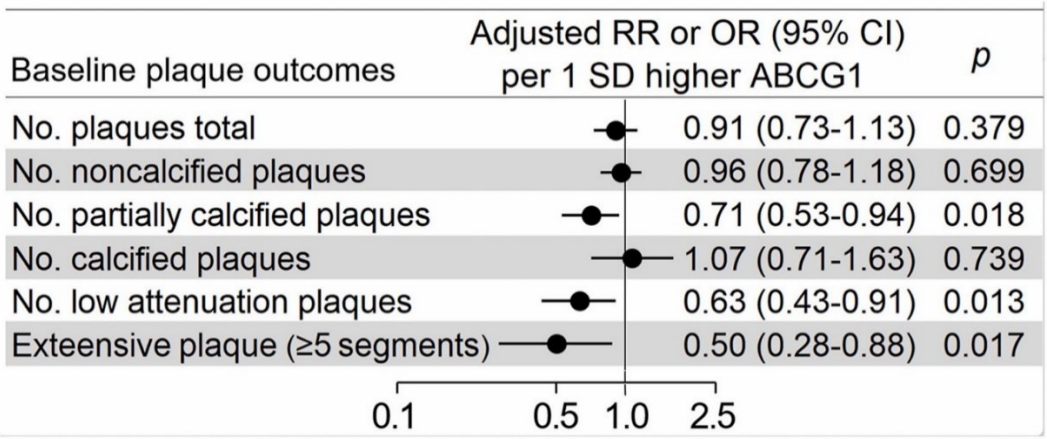

Figure 7 Association of ABCG1 HDL-CEC with baseline plaque burden in RA subjects[22].

2.2.5. Pharmacological modulation of HDL-CEC in CV diseases

There is increasing interest in developing pharmacological approaches to improve HDL function, rather than HDL-C levels, as a potential strategy to reduce CV risk, given the inverse association of HDL-CEC with CV risk.

CETP inhibitors, by inhibiting cholesteryl ester transfer protein (CETP), which normally transfers cholesterol from HDL cholesterol to VLDL or LDL, have been the first approach to improve HDL-CEC. In this context, in the TULIP randomized, double-blind, placebo-controlled Phase II trial, 364 patients were treated with placebo or TA-8995, a novel CETP inhibitor, or TA-8995 in combination with rosuvastatin[182]. The study reported a dose-related increase in HDL-CEC in patients treated with TA-8995 compared to the placebo group. In addition, a significant increase in pre-β HDL was observed, which was positively correlated with the increase in total HDL-CEC from macrophages and specifically the ABCA1-mediated HDL-CEC, but not with the increase in HDL-C[182].

Studies evaluating the impact of statins, the first-line therapy for the treatment of hypercholesterolemia, on HDL-CEC have produced conflicting results. Rosuvastatin and simvastatin significantly reduced total and ABCA1-dependent HDL-CEC, whereas atorvastatin showed no statistically significant effect on any cholesterol efflux pathway in the Phase II LY2484595 study evaluating 2 weeks of evacetrapib monotherapy or in combination with atorvastatin, rosuvastatin, or simvastatin[183]. When evacetrapib was added to statin therapy, HDL-CEC increased significantly, but this effect was attenuated compared with evacetrapib monotherapy[183]. Similarly, the addition of rosuvastatin to TA-8995 did not change HDL-CEC compared to monotherapy with TA-8995[182]. Additionally, a post-hoc analysis JUPITER evaluated the association between HDL-CEC and cardiovascular events in statin-treated patients. Treatment with rosuvastatin did not lead to a significant change in HDL-CEC at 12 months[184]. Overall, this lack of a significant impact of statins on

HDL-CEC is consistent with the modest effect of this class of drugs on HDL-C concentration and subclass distribution[185].

Tocilizumab, a humanized monoclonal antibody that acts as an IL-6 receptor antagonist, is approved in numerous countries for the treatment of adults with moderate to severe active RA[186]. After 12 weeks of tocilizumab treatment in RA subjects, we previously found that HDL-C levels were unchanged, but HDL-CEC was significantly ameliorated for the SR-BI and ABCG1 pathways with respect to baseline[187]. A recent meta-analysis showed that early control of inflammation and antirheumatic treatment in patients with RA may improve HDL-CEC, but have no impact on HDL-C levels, even if these findings and interpretations are limited by the quality and quantity of available evidence[188].

A recent review has demonstrated that another strategy to enhance HDL-CEC is related to the beneficial effects of three major apoAI-based approaches: MDCO-216, CER-001 and CSL111/CSL112. Two of them (MDCO-216 and CER-001) reduce LCAT activity, thereby impairing HDL maturation and its function in promoting cholesterol efflux. CSL112 increases LCAT activity without affecting the function of native apoAI. CSL112 significantly enhances cholesterol efflux[189] even if the Phase III clinical trial did not meet its primary efficacy endpoint of MACE reduction at 90 days (ClinicalTrials.gov NCT03473223).

The molecular mechanism underlying HDL-CEC modifications are under investigation, as well as the impact of this modification on CV reduction, which would make this parameter a new and interesting CV risk marker and therapeutic target.

2.3. Serum cholesterol loading capacity

2.3.1. LDL heterogeneity

LDL particles (size range 18-25 nm) transport cholesterol to cells and represent approximately 90% of apoB-containing lipoproteins in the blood under fasting conditions; the term LDL-C refers to the plasma concentration of LDL cholesterol for cardiovascular risk assessment and as a surrogate marker of LDL concentration[190].

Because atherosclerotic plaque growth occurs over time due to the retention of lipoprotein particles in the vascular intima, plaque size is determined by both the concentration of circulating LDL and other apoB-containing lipoproteins and the total duration of exposure, so the plaque size is proportional to the cumulative exposure to LDL and apoB-containing lipoproteins[191].

In atherosclerotic disease, oxidative stress induces changes in LDL, both phospholipid and cholesterol content, generating oxidized LDL (oxLDL) with increased affinity for macrophage scavenger receptors leading to macrophage accumulation of oxLDL and foam cell formation[192]. In

the subendothelial space, oxidative stress occurs in two phases by enzymatic and non-enzymatic oxidants, both free (single-electron) and non-free (double-electron), derived mainly from reactions catalyzed by 12/15-lipoxygenase, myeloperoxidase, nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and transition metals (heme and hemoglobin). In the first phase, two-electron oxidants such as hypochlorite and peroxynitrite modify LDL almost instantaneously, attacking mainly apoB. Free radicals do not modify apoB during this phase, forming minimally oxidized LDL (mmLDL), which retains affinity for the LDLR, activating the signaling pathway for the secretion of pro-inflammatory chemokines and cytokines[193–195]. This leads to loss of affinity of the LDLRs and recognition of oxLDLs by scavenger receptors expressed on macrophages[196]. In addition, mmLDL contributes to monocyte recruitment by stimulating the release of monocyte chemotactic protein-l (MCP-I) and monocyte adhesion by regulating the endothelial adhesion molecules E-selectin, P-selectin, ICAM-1, and VCAM-1[197]. In addition, a growing body of evidence suggests that mmLDL promotes inflammatory cell activation through TLR4, leading to a variety of proatherogenic responses, including lipid accumulation, ROS production, and cytokine secretion[198]. LDL can also be classified into several subclasses in ascending order of density and descending order of size: large (LDL-I), intermediate (LDL-II), small (LDL-III), and very small (LDL-IV) particles. The latter subclass is also referred to as small dense LDL (sdLDL) and its presence is associated with hypertriglyceridemia conditions due to increased activity of CETP, which is involved in the exchange of TG of VLDL with CE of LDL by becoming the substrate of hepatic lipase (HL), resulting in the formation of more atherogenic sdLDL[199,200]; the main atherogenicity of this class could be explained by several hypothesized mechanisms: a reduced exposure of apoB on the surface of sdLDL particles, resulting in a lower affinity for LDL receptors and, consequently, prolonged plasma retention; the small size of these lipoproteins facilitates trans-endothelial transport and entry into the subendothelial space; sdLDL are more susceptible to oxidative modification due to their reduced antioxidant content[201]. Indeed, recent studies have shown that an increase in small LDL is associated with an increased incidence of CV in patients without coronary atherosclerosis[202,203].

2.3.2. Serum cholesterol loading capacity (CLC) and CV risk

Serum lipoproteins, LDL in particular, are the principal cholesterol carriers delivering cholesterol to macrophages. Normally, the delivery of cholesterol from serum to cells is regulated by intracellular cholesterol content[204]; however as previously mentioned, in CV diseases, usually characterized by a chronic inflammatory status and/or an increased oxidative stress, lipoproteins undergo several modifications and cholesterol internalization, particularly in macrophages in the vascular wall, occurs through unregulated pathways[195].

Macrophages express a large repertoire of pattern recognition receptors (PRRs) at high densities on their surface, including so-called "scavenger" receptors, SR-A1, SR-A2, cluster of differentiation 36 (CD36), and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), that mediate the binding and uptake of oxLDL and acetylated LDL (acLDL). CD36 and SR-A have the highest affinity for oxLDL and acLDL and are responsible for up to 90 % of their uptake by macrophages in vitro[205]. CD36-mediated oxLDL uptake depends on the activation of Src and JNK kinases[206]. LDL oxidation and uptake of oxidized rather than native LDL-C by scavenger receptors on macrophages are critical steps in the formation of foam cells[195] (**Figure 8**).

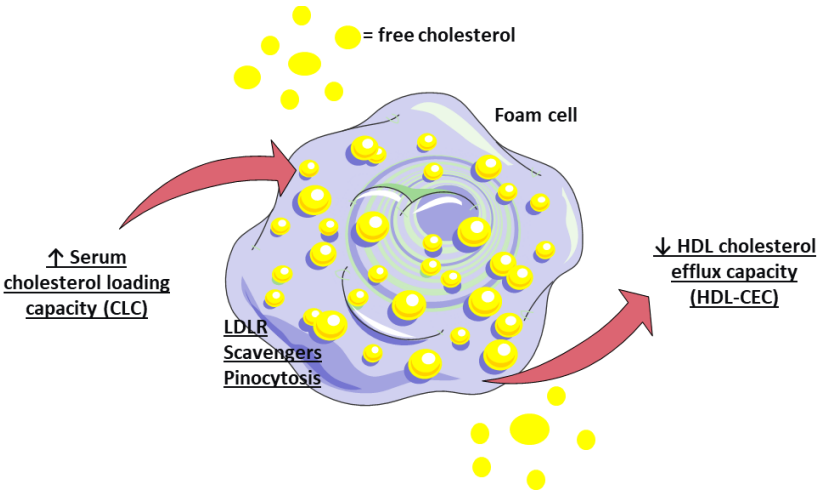

Figure 8 Schematic illustration of altered cholesterol homeostasis and foam cell formation in atherosclerosis.
Pictures have been created by combining images from Smart Servier Medical Art (https://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

In support of this, it has been reported that in apoE-/- mice, CD36/SR-A deficiency did not produce foam cells in atherosclerotic lesions and did not affect lesion size, but a reduction in the complexity and size of necrotic areas in lesions was detected, suggesting a role for CD36 and SR-A in cell death[207]. Different studies found that deletion of these receptors decreased atherogenesis, but in contrast, other in vivo studies have shown that apoE mice deficient in CD36 or SR-A do not have less atherosclerosis than wild-type apoE mice[208]. The discrepancy between results obtained in different laboratories underscores the complexity of the mechanisms of macrophage lipid accumulation and atherosclerosis[208,209]. Other scavenger receptors that have been reported to mediate internalization of oxLDL include the LOX-1, the receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX/CXCL16), the scavenger receptor expressed by endothelial cells (SREC-I), the receptor for lipoprotein type B1 (SR-BI), CD68, and the receptor for advanced glycation end products (RAGE)[208,209]. In contrast to the LDLR, whose expression on the cell surface is dependent on intracellular cholesterol levels, the expression of scavenger receptors, lacks this negative feedback mechanism and thus mediates extensive cholesterol accumulation in the macrophage. In addition,

native and some forms of modified LDL may also undergo phagocytosis and pinocytosis, thus contributing to the accumulation of lipoproteins in macrophages[210].

Inhibition of macrophage scavenger receptor activity may provide the basis for anti-atherogenic therapy. However, it may be necessary to target several classes of proteins simultaneously. Since these receptors are also involved in the clearance of microorganisms, there may be an increased susceptibility to certain infectious agents. It is also possible that inhibiting the function of scavenger receptors could have deleterious effects, as they play an important role in clearing apoptotic cells or performing other critical functions. For example, the primary function of SR-BI is to mediate reverse cholesterol transport by HDL and its deletion in apoE-/- mice causes severe atherosclerosis with evidence of plaque rupture and acute myocardial infarction, rare complications in other mouse models of atherosclerosis, despite it binds also to oxLDL[211,212].

Cholesterol loading capacity (CLC) is a recently described parameter that reflects the ability of all serum lipoproteins to deliver cholesterol to cultured human macrophages under standard conditions. Measurement of CLC involves quantification of cholesterol in cell lysates after exposure of cells to study population whole serum[213]. CLC is associated with foam cell formation[214] and has been found to be increased under conditions associated with increased cardiovascular risk[215,216].

In particular, as reported in recent studies, some conducted in our laboratory in patients with hypogonadism, a condition associated with high CV risk, lipoprotein-associated proatherogenic changes are associated with increased inflow, independently of LDL-C levels, so they may offer an explanation for the potentially increased CV risk in this population[215]. In another recent study conducted in our laboratory, CLC was associated with the risk of long-term cardiovascular events in RA subjects and with the presence of high-risk obstructive coronary plaques in nonusers of disease-modifying antirheumatic drugs, after adjustment for LDL-C levels (**Figure 9A**)[20].

This and numerous other evidence have led to the conclusion that this parameter is an index of serum atherogenicity and, therefore, to the development and evaluation of possible pharmacological interventions that can modulate and reduce this parameter in order to reduce CV risk.

2.3.3. Pharmacological modulation of serum CLC in CV diseases

The pro-atherogenic potential of serum, by the evaluation of CLC and its involvement in foam cell formation has been demonstrated Therefore, modulation of CLC, is an increasingly sought-after therapeutic strategy, with the goal of achieving an ever-lower CV risk, especially in chronic diseases, characterized by the presence of comorbidities that lead to a very high cardiovascular risk that is difficult to control.

Currently, research in this area is limited, and the evidence of a reduction in CV risk following modulation of cholesterol internalization by macrophage is indirect, due to a lack of adequate prospective studies. However, much evidence has been reported from *in vivo* studies, and many other real-world evaluations have been made in our laboratory. The effect of a pharmacological modulation was reported by our group, as already mentioned: in detail, in patients with RA, after 12 days of treatment with tocilizumab, LDL-C levels increased while CLC was reduced, underscoring an impact of this treatment on the reduction of serum pro-atherogenic potential independently of serum LDL-C levels[187]. *In vivo,* treatment of atherosclerosis-prone mice with the tumor necrosis factor α (TNF-α) inhibitor adalimumab suppressed the influx of monocytes into the arterial wall and inhibited TNF release from macrophages[217], and this seems to be reflected in humans as well, given the results reported by Karpouzas and colleagues that treatment with biologic disease-modifying antirheumatic drugs (bDMARD) decreased new plaque formation in patients with RA without coronary atherosclerosis or with early non-calcified lesions, although the intake capacity[218]. This evidence seems to find an explanation in the results of a very recent work by Karpouzas and colleagues, in which treatment with bDMARD attenuated the relationship between CLC and plaque presence and burden only in treated subjects, while in non-users a low probability of attenuation and presence of obstructive plaque in coronary segments was reported (**Figure 9B**)[20].

This evidence, although limited, suggests that modulating the ability of serum to promote CLC in macrophages to improve lipoprotein function, could be a possible adjunctive strategy for CV protection and risk reduction.

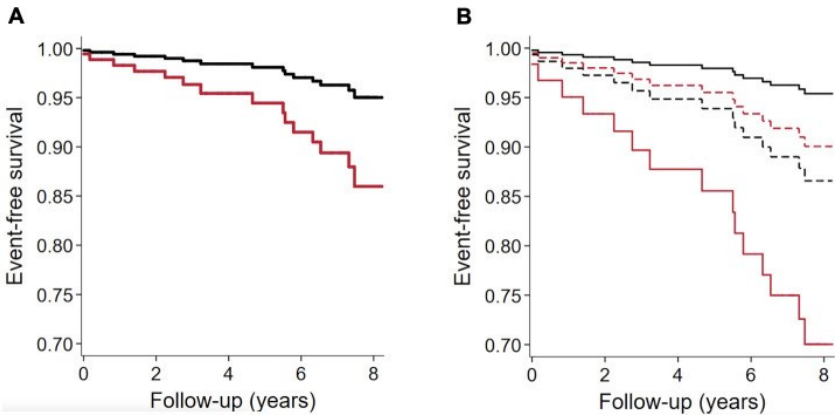

Figure 9 Association of serum CLC with CV event risk in RA subjects. **(A)** CLC association with greater CV risk after adjustments for CV risk score and number of coronary segments with plaque. **(B)** CLC association with higher CV event risk in bDMARD nonusers. Solid lines represent bDMARD nonusers and dashed lines represent bDMARD users[20].

2.4. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)

2.4.1. Role of PCSK9 in the regulation of cholesterol homeostasis

PCSK9 was discovered in neurons in 2003 and originally named neural apoptosis-regulated convertase (NARC-1), as mentioned before; it belongs to a group of serine proteases that lead to

the hydrolysis of peptidic bonds[219]; subsequently, interest in this protein increased due to the observation of Abifadel et al. who identified an association between genetic mutations in the gene encoding PCSK9 and autosomal dominant hypercholesterolemia[220]. The discovery of this convertase allowed the identification of an important new modulator of cholesterol homeostasis and stimulated intense research activities. Studies on in vivo and in vitro experimental models have allowed to better elucidate the relationship between this extracellular protein, the expression of LDLR and, consequently, LDL cholesterolemia.

PCSK9 is a 692 amino acid protein that belongs to the proprotein convertase family[221], which includes nine serine proteases that are responsible for the proteolytic maturation of secreted proteins such as hormones, cytokines, and growth factors, as well as membrane proteins such as receptors and integrins. Convertases are characterized by the presence of an active site capable of catalyzing the enzymatic hydrolysis of peptides[222].

The human PCSK9 gene is located on chromosome 1p32.3, and consists of 12 exons. PCSK9 is mostly expressed in the liver, small intestine, kidney and central nervous system. PCSK9 is synthesized as a zymogen precursor of approximately 75 kDa (pro-PCSK9). The crystal structure of the human protein revealed the presence of a signal peptide (amino acids 1-30), a pro-domain (amino acids 31-152), a catalytic domain and a C-terminal domain[223]. The pro-PCSK9 precursor undergoes self-catalytic cleavage at position 152 in the endoplasmic reticulum, releasing the N-terminal residue and the mature protein of approximately 60 kDa. In particular, unlike all other convertases, the pro-domain of PCSK9 remains attached to the C-terminal domain; the formation of this complex allows the movement of mature PCSK9 across the endoplasmic reticulum[224] (**Figure 10A**).

The recognition of the target by PCSK9 is achieved by the binding of the catalytic domain to the epidermal growth factor A (EGF-A)-like sequence that is present in receptors that belong to the LDLR family. Once bound to the EGF-A domain, the PCSK9-LDLR complex is internalized by clathrin-mediated endocytosis[225]. The acidic pH characteristic of late endosomes enhances the binding between PCSK9 and EGF-A, and this strong interaction inhibits dissociation and recycling of the receptor at the cell membrane, leading to degradation of the complex[226].

The binding of PCSK9 to LDLR results in altered LDL-C levels, as is clearly demonstrated by both general population correlation studies and genetic evidence. The ARIC (Atherosclerosis Risk in Communities) Genetic Study identified how mutations in the PCSK9 gene are reflected in plasma LDL-C levels and cardiovascular disease[227]. The study enrolled 13,761 subjects and analyzed PCSK9 mutations in 9,524 Caucasian subjects and 3,362 black subjects; 2.6 % of the latter were found to carry nonsense PCSK9 mutations. These mutations were associated with an average 28 % reduction in LDL-C and an 88% reduction in cardiovascular risk. Similarly, 3.2 % of Caucasian subjects had a mutation in the PCSK9 gene that was associated with a reduction in both LDL-C (- 15 %) and CV risk

(- 47 %)[227]. The results of this study have not only established the relationship between PCSK9 and LDL-C, but it has also shown that a moderate and sustained reduction in LDL-C, caused by the PCSK9 mutation, is associated with a very significant reduction in CV risk, even in a population with a high prevalence of lipid-independent CV risk factors.

Thus, PCSK9 perturbs lipid metabolism and alters circulating lipid levels, which is the most obvious mechanism by which PCSK9 is associated with atherosclerosis, but the proatherogenic effect of PCSK9 is not solely due to its effect on plasma apoB concentrations. PCSK9 is expressed at the level of SMCs and, to a lesser extent, endothelial cells[228]. In addition, PCSK9 is found at the level of atherosclerotic plaques in areas that partially overlap those occupied by SMCs[228,229]. From a purely functional point of view, PCSK9 released by SMCs could influence, in a paracrine manner, the expression of LDLR on the cell surface of macrophages[228]. In this regard, another extrahepatic mechanism of PCSK9 was recently described by our research group, which demonstrated that recombinant human PCSK9 inhibits cholesterol efflux mediated by the membrane transporter ABCA1 in macrophages, independently of LDLR[139].

As a regulator of cholesterol homeostasis, the activity of PCSK9 has also been observed in the brain, where it has been shown to regulate receptors involved in neuronal cholesterol transport, resulting in the degradation of LDLR, LRP1, VLDLR, and apoER2[230], underscores the role of this protein in the pathogenesis of AD[231].

These observations have stimulated intense research on PCSK9 over the past decade, making this protein one of the most promising targets for developing new therapies for the treatment of hypercholesterolemia[232,233] (**Figure 10B**), and stimulating future research for the treatment of neurodegenerative diseases.

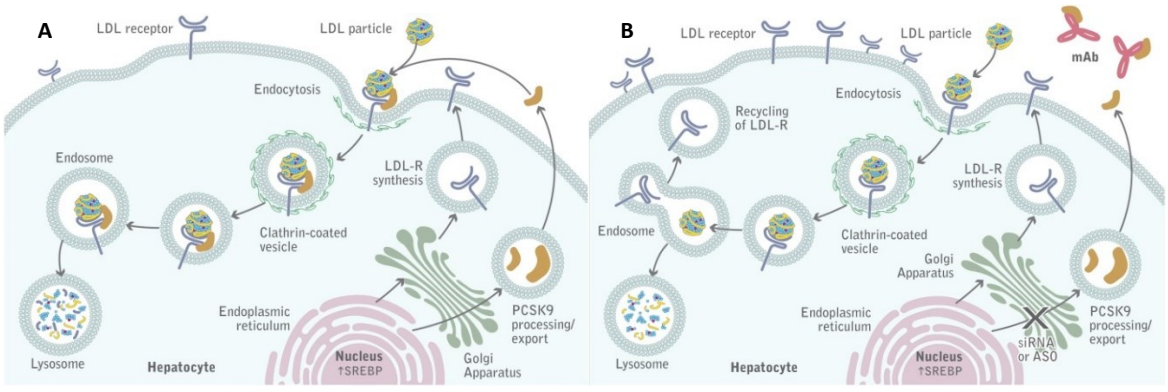

Figure 10 (A) PCSK9-mediated degradation of LDLR; **(B)** PCSK9 inhibition by monoclonal antobodies[234].

2.4.2. Pharmacological modulation of PCSK9 in CV diseases

As mentioned before, PCSK9 has become a potential therapeutic target for a variety of diseases due to its multiple functions in regulating cholesterol metabolism at the level of different body regions;

foremost, PCSK9 inhibition is a therapeutic strategy for the treatment of hypercholesterolemia and atherosclerosis, in which it plays a synergistic or substitutive role for statin and other hypolipidemic drugs; more recently, PCSK9-i have potential application in the treatment of sepsis, some tumors, viral infections, and other diseases[72].

Mechanistically, the success of PCSK9 inhibition strategies is generally attributed to three strategies targeting the pro-protein. Specifically, the first strategy is to directly or indirectly prevent the binding of PCSK9 to LDLR on the cell surface with an antibody, peptide, or small molecules. The second strategy is to interfere with the maturation and secretion process or biological function of PCSK9. The third strategy is to develop inhibitors of the synthesis and expression of PCSK9 at the mRNA or protein level[235].

Based on these major inhibition strategies, the major inhibitors of PCSK9 can be divided into four categories:

1) Monoclonal antibodies (mAb) inhibitors, such as Alirocumab (Regeneron, New York, NY, USA; and Sanofi, Paris, France) and Evolocumab (Amgen, Los Angeles, CA, USA). PCSK9 mAbs block the interaction between PCSK9 and LDLR, inhibiting the degradation process of LDLR[72]. From the numerous clinical trials conducted to evaluate the primary and secondary outcomes of this class of drugs, the FOURIER and ODYSSEY trials, it is reported that the use of mAbs leads to an average reduction in cholesterol levels of 50 – 60 %, both as monotherapy and when administered in combination with a statin. This reduction in LDL levels, as shown in numerous epidemiologic studies and meta-analyses, is proportional to a reduction in relative cardiovascular risk of about a 40 - 50 %[236].

2) Nucleic acid-based drugs, such as antisense oligonucleotides (ASO), short-interfering RNA (siRNA), as the well-known Inclisiran (Alnylam, Cambridge, MA, USA), and CRISPR/Cas9 gene editing systems[72]. ASOs bind to the mRNA of the target gene PCSK9 through Watson-Crick base pairing interactions and limit the expression of the target gene[237]. SiRNA interferes with mRNA degradation and inhibits PCSK9 gene expression, thereby inhibiting the synthesis of the protein[238].

3) Small molecules block the biological activity of PCSK9 by preventing binding to LDLR, disrupting the subsequent degradation steps, and increasing receptor cycling and LDL uptake. Although only two small molecule PCSK9 inhibitors, CVI-LM001 derived from corydaline and DC371739 from berberine[239], are currently in Phase II (Trial NCT04438096) and Phase I (Trial NCT04640012) respectively, there has been a steady increase in available data on small molecule PCSK9-i in recent years given the many advantages of this formulation, most notably a lower cost and a better tolerated oral route of administration than the intravenous route used for the more common PCSK9-¡[235,240].

4) Vaccines, such as the L-IFPTA+ vaccine, which induced the generation of functional and safe antiPCSK9 antibodies as demonstrated in monkeys, can inhibit circulating PCSK9 activity[241] (**Table 1**).

Approach	Mode of action	Advantages	Disadvantages	Agent	Stage
Monoclonal antibodies (mAbs)	Blocking interaction with LDLR or neutralizing PCSK9 activity.	Efficient and safe High specificity Low toxicity	Short shelf-life compared with siRNA High cost Parenteral administration	Evolocumab Alirocumab Bococizumab LY-3015014 RG-7652	Approved-2015 Approved-2015 Phrase III (Discontinued) Phase II Phase II
Small Peptides	Blocking interaction with LDLR	Cheap	Instability in plasma Parenteral administration	Pep2-8 MK-0616	Phase I Phase II
Antisense oligonucleotides	Silence mRNA Leading to mRNA degradation	High specificity	High production costs Parenteral administration	SPC5001 BMS-844421 Civi-007	Phase I (Discontinued) Phase I (Discontinued) Phase I
siRNA	Target PCSK9 mRNA and arrest translation	Long-acting Safe Less-frequent dosing	Parenteral administration	Inclisiran (ALN-60212)	Phase III
Adnectin	Blocking interaction with LDLR	High binding affinity Relative cheaper production cost	Short plasma half-life	BMS-962476	Phase I
Anticalin	Blocking interaction with LDLR	Antibody mimetic but smaller than mAbs, Mass production at low cost	Design and screening	DS-9001a	Phase I (Discontinued)
Vaccine	Immunization against the body's own PCSK9 to produce autoantibodies	High immune response against PCSK9 without side effects Long-term effects	Possible irreversibility Autoimmune response	AT04A& AT06A	Phase I Phase I
CRISPR	Genome editing disrupting *PCSK9* gene	Permanent reduction in LDL-C levels	Potential off-target mutagenesis	Academic project and AstraZeneca	Preclinical
Small molecules	preventing binding to LDLR	Cheap Oral administration	High likelihood of off-target	CVI-LM001 DC371739	Phase II Phase I

Table 1 Main pharmaceutical approaches targeting PCSK9, modified[235].

Currently, there are two commercially available mAbs, Alirocumab and Evolocumab, each with trial data supporting a reduction in cardiovascular risk[62,63]. These drugs are administered every 2 weeks or monthly by subcutaneous injection. PCSK9-i have been widely used in the secondary prevention of atherosclerotic cardiovascular disease, but their use is also indicated in patients with genetic disorders such as hypercholesterolemia with very high LDL-C levels[11,242].

Current cardiovascular guidelines recommend PCSK9 inhibition in high-risk patients whose LDL-C levels didn't reach the therapeutic target even under the maximum tolerated statin therapy and usually after adding ezetimibe in combination. However, the cost of these therapies remains a major issue for the use of PCSK9-i, although recent formulation with a lower price and a simpler dosage

may lead to increase the use of these drugs[243]. New PCSK9-i under investigation will further expand the armamentarium for the prevention of cardiovascular disease.

3. Role of lipids, lipoproteins and PCSK9 in the evaluated clinical settings

3.1. Role of lipids, lipoproteins and PCSK9 in FH

Low levels of HDL-C have been reported in FH in numerous studies[244]. However, assessment of HDL quality and function may be important in patients with FH as they may be both measures of atherosclerotic burden and potential therapeutic targets. Several studies indicate abnormal HDL function in FH patients[244]. Specifically, HDL-CEC has been shown to be independently and inversely associated with the presence of CV diseases (odds ratio [OR] for 1 standard deviation increase [SD] 0.95; 95 % confidence interval [CI], 0.90-0.99; $p = 0.0260$), the presence of corneal arch (OR for 1 SD increase: 0.98; 95 % CI, 0.90-1.00; $p = 0.0306$) and Achilles tendon thickness ($p < 0.001$) in 227 patients with heterozygous FH under treatment with statins[245]. Cholesterol efflux also depends on transporter expression levels on macrophages' plasma membrane. In this regard, significantly lower expression of ABCG1 was observed in peripheral blood mononuclear cells (PBMCs) from HoFH patients ($n = 7$) and patients with HeFH ($p = 0.006$) ($n = 7$) compared with age- and sex-matched healthy controls ($p \leq 0.015$); in addition, a borderline significant reduction was observed for ABCA1 ($p = 0.064$) and SR-BI ($p = 0.085$) in HoFH patients compared with healthy controls[246]. Few studies have investigated alterations in HDL-CEC in patients with FH. In one study, HDL2 particles obtained from patients with FH ($n = 12$) showed a significantly reduced ability to mediate cholesterol efflux from macrophages via SR-BI (- 50 %; $p < 0.001$) or ABCG1 (- 21 %; $p = 0.0009$) compared to age- and sex-matched normolipidemic healthy subjects ($n = 12$)[247]. Thus, there is evidence to suggest that a decreased cholesterol efflux capacity was associated with increased risk of CV diseases in HeFH patients, but the type of pathogenic mutation (loss-of-function of LDLR or gain-of-function of PCSK9) has not emerged as a significant determinant, after a logistic-regression analysis adjusted for risk factors[245].

The role of LDL in FH is well established. In these patients, a high concentration of LDL-C has been detected since birth, ranging from 8 to 15 mmol/L in HeFH and from 12 to 30 mmol/L in HoFH. An increase in LDL-C inevitably leads to early atherosclerosis development and, subsequently, to a premature death[26]. Foam cells, resulting from the accumulation of LDL cholesterol by macrophages, are recruited into the xanthomatous lesion[248]. Xanthomas are pathophysiognomic signs of FH. Histologically, xanthomas consist mainly of collagen and foam cells[249]. The lipid component is

further subdivided as follows: 55 % free cholesterol, 28 % esterified cholesterol, and 13 % phospholipids; unesterified cholesterol tends to accumulate at the extracellular level, while esterified cholesterol is equally distributed between the intracellular and extracellular compartments[250]. Experiments with technetium-labeled LDL suggest that there is a continuous exchange of cholesterol between plasma and xanthomas, making it more likely that the lipid component is derived from serum rather than local synthesis[251]. There is also a different distribution of LDL within xanthomas: mainly oxLDL were found in the intracellular compartment. It could be hypothesized that plasma LDL remains trapped in the matrix of collagen and glycosaminoglycans, where it is exposed to oxidative processes that convert it to oxidized LDL, which thus undergoes phagocytosis by macrophages, leading to foam cell formation[252,253]. Thus, this pathophysiological process is similar to that of atherosclerotic plaque formation.

Indeed, xanthomas and atherosclerotic plaques have a similar composition, both consisting of a connective matrix containing macrophages transformed into foam cells[252].

PCSK9 levels may vary in individuals with FH depending on the specific genetic mutations involved, but high PCSK9 levels have been associated with higher cardiovascular risk, while decreased PCSK9 activity is associated with lower LDL-C levels and reduced risk[254]. Mutations in PCSK9 have been identified as a cause of autosomal dominant FH due to the known role of PCSK9 in regulating cholesterol homeostasis. Specifically, gain-of-function (GOF) and loss-of-function (LOF) mutations in this gene can affect individuals with FH. To date, GOF mutations in PCSK9 have been found to be most associated with hypercholesterolemia and CV diseases[255]. Numerous mutations in PCSK9 have been identified, but only a few have been detected as relevant for molecular diagnosis of FH, contrary to the molecular landscape of the LDLR and APOB genes[71].

3.2. Role of lipids, lipoproteins and PCSK9 in AAA

Dysregulation of lipid metabolism plays a prominent role in cardiovascular disease, but its involvement in the development of AAA is still unclear, especially in terms of aetiopathogenetic mechanisms[84]. The relationship between AAA and atherosclerosis is not clear; there is some epidemiological correlation between the two processes: because of some common risk factors, and the involvement of lipid metabolism, the two diseases often occur together in an unclear manner[256]. Several studies have shown that dyslipidemia causes AAA: a prior research from Malmö Preventive Study showed increased TC and TG in AAA subjects compared to controls[257]. Genetic analyses have directly correlated AAA with total and LDL cholesterol levels, but not with triglycerides and HDL[258], even if Singh K et al. found that HDL-C concentration was lower in patients with AAA than in control subjects in a population of 6386 subjects[259]. This and other studies have supported the idea that

high plasma levels of TC and LDL-C and low levels of HDL-C would be the major risk factors in this condition[259,260].

Beyond lipoprotein levels, because of the relevance of lipoprotein function (see paragraphs 2.2.4 and 2.3.2), targeted studies have also shown an alteration in HDL function related to specific factors such as the anti-oxidant property[261], and the anti elastase activity, reporting a reduction of alpha1-antitrypsin content in HDLs[262].

An HDL proteomics study indicated that molecules involved in oxidative stress and immune activation are the most specifically associated with AAA, in particular, peroxiredoxin-6 as a potential new biomarker of the disease[263]. Also, genetic studies indicate that specific polymorphisms of the cellular cholesterol transporter ABCA1 are associated with AAA[264]. HDL in AAA also showed a loss of vasculoprotective properties: post-translational modifications detected in plasma of AAA patients, particularly oxidation of apoAI, could be the responsible for the loss of their main function, namely cholesterol efflux capacity[265].

However, there is currently no direct evidence of HDL-CEC impairment in AAA. Altered HDL-CEC has been indirectly demonstrated in AAA, i.e., through analysis of transporters' tissue expression at the level of the aneurysmal aortic wall, but this impaired HDL function would then be due to specific cell abnormalities and not to HDL dysfunction[266]. To date, recent works report an impairment in HDL-CEC, evaluated on murine macrophages treated with cyclic adenosine monophosphate, which promote ABCA1 expression, and T0901317, which enhances ABCA1/ABCG1 expression. They observed ABCA1-mediated HDL-CEC higher in controls (14.23 ± 0.51 %) compared to AAA subjects (10.80 ± 0.62 %) ($p < 0.001$), and an ABCA1/ABCG1- mediated HDL-CEC of 25.29 ± 0.56 % in controls versus 21.39 ± 1.00 % in AAA patients ($p = 0.002$), results probably due to reported low levels of plasma apoAI and pre-β HDL particles in AAA subjects[267].

On the other hand, LDL-C, due to their proven role in the process of atherothrombosis and their relationship to a higher-risk of cardiovascular events levels, were found to bear the most significant association with AAA growth rates[268]. In addition, the presence of small and dense LDL may be a common trait in patients with AAA[269]. The pro-atherogenic potential of LDL also increases under conditions characterized by increased oxidative stress, with the formation of oxLDL, even if the influence of oxLDL on enlargement and rupture of AAA was demonstrated negligible from the results of Gutowski et al.[270].

Two SNPs found in the lp(a) gene may be involved in AAA, resulting in higher levels of circulating lp(a) in AAA subjects[84]. In the ARIC study, plasma levels of lp(a) correlated with the incidence of AAA independently of other risk factors and LDL cholesterol and triglyceride levels[271]. Lp(a) would be responsible for endothelial dysfunction in AAA, by increasing expression of MCP-I, phospholipid oxidation, and SMC alterations[272].

Mutations in genes encoding for LDL receptors (LRP1 and LDLR), also appear to be associated to AAA: functional analyses have shown that the mutation in LRP1 gene seems to alter an SREBP-1 binding site, while the function of the mutation in LDLR gene remains unclear[84].

To date, no data are available on the correlation of LDL functional modification and AAA.

A recent study has shown that PCSK9 gene expression is higher in the aneurysm wall of patients with AAA, suggesting an implication of PCSK9 in the loss of structural integrity of the aorta and subsequent imbalanced vasoconstriction, given the reported role of PCSK9 expressed in aortic wall smooth muscle cells in developing acute aortic dissection (AAD)[273]. Single-nucleotide polymorphisms in PCSK9 gene have been associated with AAA[274]. This finding seems to be confirmed by the results of an *in vivo* study, where a PCSK9's gain-of-function mutations induced an aneurysm formation with an increase in circulating cholesterol, by augmenting AngII-induced AAAs in C57BL/6 mice[275].

3.3. Role of lipids, lipoproteins and PCSK9 in AD

As mentioned before, dysregulation of lipid homeostasis is correlated with neurological disorders and neurodegenerative diseases such as AD. This is mainly due to the fact that lipids are fundamental cellular components and play an important role in brain physiology: in fact, it is an essential structural component for cellular membrane and myelin and a precursor of steroid hormones and bile acid synthesis, but also a required component for synapse and dendrite formation[276]. Brain is the most lipid-rich organ, it contains 20 % of the whole body's cholesterol, and cerebral lipids percentage amounts for at least 50 % of dry brain weight[276,277]. The presence of intact BBB separates cholesterol metabolism in the brain from the rest of the body and it is well known that neuronal cells provide the maintenance of cholesterol homeostasis by a feedback mechanism that balances biosynthesis, import, and excretion[276].

Several epidemiologic studies suggest that HDL may play a role in AD, but the evidence remains controversial. Most studies suggest an inverse association between higher plasma HDL-C levels and reduced risk of dementia and AD, as well as better cognitive function and typical AD-associated trait[278,279]. However, this association appeared only in subjects under 70 years[280]. Some research suggests that higher levels of apoAI correlate with improved cognitive scores and lower risk of AD[281]. However, other studies have found either no association[282] or even a positive correlation between plasma HDL-C level and AD severity[283].

Understanding how plasma HDL could be involved in AD progression remains still unclear. In patients with AD, abnormalities in plasma HDL are observed, including altered structure, an increase in apoCIII, and a loss of S1P[284]. These abnormalities interfere with HDL's ability to remove cholesterol,

particularly affecting specific pathways responsible for cholesterol efflux. In addition, HDL from Alzheimer's patients exhibits pro-oxidant characteristics and impaired anti-inflammatory properties, in part due to reduced activity of a key molecule, lipoprotein-associated phospholipase A2, involved in CNS redox processes[285].

With regard to the central nervous system, CNS HDL particles, also known as “HDL-like-particles” as they have a size and density similar to plasma lipoproteins, play a critical role in regulating brain cholesterol balance, potentially affecting neurodegeneration in AD. These particles mediate the transport of cholesterol from astrocytes to neurons since adult neurons progressively lose the capacity to synthesize cholesterol[286]. Thus, alterations in brain HDL may disrupt this balance, leading to neuronal cholesterol depletion and affecting AD progression[287].

This process begins with lipid-free apoE secreted by astrocytes. This initiates a transport involving specific membrane transporters such as ABCA1 and ABCG1, which are critical for final cholesterol delivery to neurons, by promoting the lipidation of apoE, the main constituent of CNS-HDL-like lipoproteins[287].

The APOE gene, found on chromosome 19, encodes for apolipoprotein E, a 34-kDa glycoprotein consisting of 299 amino acids, the N-terminal end of which serves t to form a bond with the target receptor, while the C-terminal portion binds molecules of a lipid nature; the protein exists in three forms: apoE2, apoE3, apoE4, which have cysteine and arginine differentially in position 112 and 158. The isoform apoE4 is characterized by the presence of two arginines at positions 112 and 158 and emerges as the isoform with the lowest affinity for lipids and the graet propensity to self-aggregate[113]. The presence of apoE4 has been evidenced in 60 % of AD subjects, and it is currently believed to be the main genetic factor for the development of AD, even if the molecular mechanisms responsible for this association are not completely understood. ApoE4 is considered an important risk factor for Alzheimer's disease because of its ability to: increase β-amyloid deposition and promote plaque formation; decrease β-amyloid clearance; increase tau protein phosphorylation; dysregulate metabolic homeostasis; destabilize microtubules, leading to synaptic dysfunction; and regulate neuroinflammation[288]. As reported, the cleavage of the C-terminal portion of apoE4 results in the formation of fragments that cause neurotoxicity in cellular cytosol; the target of these fragments is cytoskeletal components, so an accumulation of them results in hyperphosphorylation of τ protein, inflammatory damage, formation of neurofibrillary tangles, exacerbating behavioral and cognitive decline[289].

ApoE4, differently from the other apoE isoforms, also promotes Aβ oligomerization and deposition by binding Aβ: this has been confirmed by analyses of the brains of patients with AD[290].

ApoE lipidation is mediated by ABCA1 activity expressed on the membrane of astrocytes; in presence of apoE4, ABCA1-mediated efflux is lower compared to other isoforms, probably because

apoE4 is responsible of the reduction of ABCA1 membrane recycling, as apoE4 induces greater ARF6 protein expression, a mechanism that favors the trapping of ABCA1 in endosomes and decreased its recycling to the plasma membrane, and its ability to lipidate apoE, as shown in a recent study[291]. This finding is in line with the results of further studies, some conducted in our laboratory, in which the ABCA1- and ABCG1-mediated efflux is reduced in individuals with mild cognitive impairment and in those with Alzheimer's disease[292,293]. A recent study conducted in our laboratory might suggest that this impairment could probably also be due to Aβ and phosphorylated tau deposition burden in AD, as ABCG1 CSF-CEC inversely correlated with the Aβ deposition in the insoluble form, and ABCA1 CSF-CEC was inversely correlated to phosphorylated tau levels, a marker of neurodegeneration[294].

Other apolipoproteins found to be involved in AD pathophysiology: reduced levels of apoAI in AD CSF have been observed, correlating with disease progression, and a genetic mutation of the apolipoprotein J (clusterin, CLU) were reported to be a well-known risk factor for AD cause its important role in the deposition and clearance of Aβ[295].

Cholesterol efflux alterations in AD patients may also be related to changes in the composition of HDL-like particles; in fact, the accumulation of triglycerides or the reduction in phospholipid content in the particles could be due to an altered function of the lecithin-cholesterol acyltransferase (LCAT) enzyme; this leads to an alteration of HDL maturation, resulting in a reduced affinity for the transporter ABCG1 and thus less promotion of cellular cholesterol efflux[296,297]. These and other evidence support the theory that HDL involvement in AD etiology and progression goes beyond the simple evaluation of HDL levels[297].

Among the key regulators of cholesterol homeostasis, some important factors also play a crucial role in the pathogenesis of AD. It is in fact well known that PCSK9, beyond its relevance regulating lipid metabolism, has been shown to play a crucial role in pathogenic processes also in the CNS, even if the impact in the brain is not completely understood[298]. In fact, as mentioned below, PCSK9 is mainly expressed in the liver, but it is also present in brain cells[299]. In the brain, PCSK9 targets LRP1, LDLR, and apoER2[230], the main receptors involved in the internalization of cholesterol, produced by astrocytes, in neurons, suggesting that the mechanism of action of PCSK9 in the central system is in part comparable to that in the peripheral compartment; this hypothesis is supported by the results of an *in vitro* study conducted on HEK293 cell line in which LDLR, VLDLR, apoER2 gene expression was substantially reduced in PCSK9 overexpressing cell compared to control cells[225]. LDLR is directly involved in the pathogenesis of AD, because of its role in neuronal structure and synaptogenesis, Aβ clearance, and synaptic plasticity[300]. PCSK9 could also affect Aβ accumulation through inactivation of the lipoprotein receptors LRP1 and CD36, the two main responsible for Aβ clearance and uptake, respectively, by neurons, microglia and astrocytes[230]. PCSK9 degrading

apoER2 also induces neuronal death, as shown by numerous *in vitro* studies, since this receptor is responsible of neuronal survival[301,302]; the pathogenesis of AD may include this pro-apoptotic effect of PCSK9.

AIM

Circulating lipoproteins are currently considered to play a very important role in cardiovascular disease. High-density lipoproteins (HDL) are considered atheroprotective because their role to remove the excess of cholesterol from peripheral tissues by promoting the reverse cholesterol transport (RCT). In this process, HDL act as acceptors of cholesterol from macrophages of the arterial wall, carrying it to the liver for excretion into the bile and feces[303]. Despite several epidemiological studies have shown an inverse relationship between plasma HDL cholesterol (HDL-C) levels and cardiovascular (CV) diseases risk, pharmacological intervention studies aimed at raising HDL-C levels have not generally supported a beneficial effect on CV outcomes[304]. This statement is reinforced by genetic studies showing that neither rare nor common genetic variants associated with HDL cholesterol levels have been strongly linked to coronary disease risk[305].

In the last years, a new concept emerged: the importance of HDL quality as an index of functionality, that may depend on HDL proteins content and morphology, but not necessarily on their concentration in plasma. The idea is that the quality, rather than mere plasma concentration, is a better predictor of HDL atheroprotective potential and thus CV risk.

HDL are responsible for several atheroprotective functions, the most important of which, and the one of greatest interest to us is the capacity of HDL to promote cholesterol efflux from macrophages (HDL-CEC). HDL-CEC could be measured with an in vitro standardized, radio-based technique that allows to distinguish among the various mechanisms of efflux through the different pathways involved[177]. HDL-CEC inversely correlates with CV, independently of HDL-C levels[179], and may be positively modulated by pharmacological or nutraceutical interventions interventions. In this regard, our group found that in patients with rheumatoid arthritis (RA) treated with methotrexate (MTX) and Adalimumab there was an increase in serum HDL levels and a transient increase in SR-BI-mediated HDL-CEC compared to RA patients treated only with MTX[213]. More recently, apoAI-based approaches (MDCO-216, CER-001 and CSL111/CSL112) are being developed for which an additive effect on HDL-CEC has been demonstrated[189].

On the other hand, Low-Density Lipoproteins (LDL) are atherogenic because of their capacity to be loaded by macrophages, which may result in the formation of foam cells, a hallmark of atherosclerosis. The correlation between circulating LDL-C levels and CV risk is very strong. Not only the quantity of these lipoproteins appear to be relevant, but also the function of LDLs plays a crucial role in the CV risk, while the higher pro-atherogenic potential would be attributable to specific subclasses of LDL such as oxidated and the small dense LDL, characterized by a specific protein composition, able to stimulate vascular damage and thrombosis. The pro-atherogenic potential of LDL also increases in conditions characterized by acute oxidative stress, resulting in the production of oxidized LDL (oxLDL), which can induce foam cell formation and inflammation in the vascular intima. An indicator of LDL functionality is the serum Cholesterol loading capacity (CLC), a parameter

that is the net result of several factors, including LDL levels and function, but also HDL function, and is a measure of lipoproteins' ability to accumulate cholesterol in macrophages. Thus, this parameter represents an index of serum atherogenicity. Serum CLC is not always related to the concentration of LDL-C, but rather to the abundance of specific LDL subgroups with a higher atherogenic potential, correlated to the oxidative status of these lipoproteins[215]. Indeed, CLC is raised in pathological conditions associated with higher CV risk and may be modulated by hypocholesterolemic interventions[306]. For example, in RA patients in treatment with Tocilizumab, we have previously demonstrated that serum LDL cholesterol levels were significantly increased ($p = 0.043$), while CLC was reduced after 12 weeks of treatment (− 0.95 %)[187].

In the context of cholesterol homeostasis, beyond lipoprotein functions, the proprotein convertase subtilisin/kexin type 9 (PCSK9) plays a critical role. PCSK9 regulates cholesterol homeostasis by degrading the hepatic low-density lipoprotein receptor (LDLR); when PCSK9 binds the LDLR in the extracellular surface, as a result of this binding, it prevents the LDLR recycling, caused by the receptor enzymatic degradation. PCSK9, by promoting LDLR degradation, affects plasma concentration of LDL-C. Indeed, PCSK9 activity increases circulating LDL concentration favoring foam cell formation, a process directly related to the pathogenesis of atherosclerosis[307]. This information underlines the importance of PCSK9-i that by inhibiting the activity of PCSK9, reduce LDL-C levels, with a decrease of myocardial infarction, stroke, and coronary revascularization[308]. Beyond the specific role in modulating plasma lipids, it has been demonstrated that PCSK9 also has a direct effect on macrophages by modulating the activity of ABCA1, but also that of scavenger receptors, promoting foam cell formation, showing pleiotropic effects and directly influencing the pathogenesis of atherosclerosis[139]. This evidence opens to new potential anti-atherosclerotic properties of PCSK9 inhibition, independently of the regulation of LDL-C levels.

When we talk about serum functional lipid profile we refer to these three main components that synergistically are involved in the regulation of the cellular cholesterol homeostasis.

Based on these premises, the first aim of this study was to identify possible disturbance of the serum functional lipid profile in different clinical settings associated with a high cardiovascular risk as well as in neurodegenerative diseases, by measuring the HDL-CEC and the serum CLC. In addition, another objective of this study was to evaluate whether the possible alteration of serum functional lipid profile in different clinical settings may be related to plasma levels and/or lipoprotein composition and to cardiovascular risk parameters in order to better define the possible diagnostic role of HDL-CEC and CLC. Within this aim we included in this study subjects affected by Familial Hypercholesterolemia (FH), Abdominal aortic aneurysm (AAA), and Alzheimer's Disease (AD). Even if these diseases seem to be very different from each other, in all of these dyslipidemia plays a very crucial role, since it is an important risk factor for the development of all these diseases.

Secondly, the project aimed to evaluate how a pharmacological intervention, with PCSK9 as target, can affect serum functional lipid profile, an essential property in the maintenance of cholesterol homeostasis. For this reason, we evaluated the potential changes in both HDL-CEC and serum CLC after 6 months of treatment with PCSK9 monoclonal antibodies inhibitors as pharmacological treatments in patients with FH.

Overall, the main final objective of this study was to evaluate whether the assessment of the serum functional lipid profile could help in the future to better stratify patients according to CV risk and if its modulation ends up in a favorable effect on the CV profile.

MATERIALS AND METHODS

1. Subjects and assessment of key biochemical parameters

1.1. Familial Hypercholesterolemia (FH) subjects with Achilles tendon xanthoma (ATX)

Subjects included in this work from this clinical setting are included in the LIpid transPort disorders Italian GEnetic Network (LIPIGEN) project, within which clinical and biochemical data were collected, and molecular analysis of the main FH-causing genes was performed. 349 hypercholesterolaemic subjects (mean age 43.1 ± 20.5; males 163, females 186) were enrolled at the Medical Department of the University of Padua for a clinical suspicion of FH based either on DLCN score or on physician's judgment, all participants provided written informed consent.

Among these, we selected 45 patients (mean age 4.8 ± 15.6; males 16, females 29) with the following distribution of FH-caused gene mutations:

- 35 subjects with mutations in the LDLR, apoB, PCSK9 or LDLRAP1 genes (78 %);
- 4 subjects with polygenic hypercholesterolaemia;
- 6 any pathogenic variant: they had a definite clinical diagnosis of FH according to the DLCN, but no pathogenic variant was identified. They were probably carriers of yet-unidentified genetic mutations associated with the hypercholesterolemic phenotype; this finding is in line with the prevalence reported in published studies of unknown mutations associated with hypercholesterolemia, reported in about 20% of FH subjects[309,310].

For each patient, we had demographic and clinical data according to a standardized case report form. Specifically, we examined the familial and personal medical history of hypercholesterolemia and atherosclerosis CV disease for the clinical diagnosis of FH according to the DCLN score. We also considered the other CV risk factors (i.e., smoking habit, hypertension, diabetes) and any ongoing type of lipid-lowering treatment. On the physical examination, we calculated the BMI.

Achilles tendon xanthoma (ATX) was evaluated with ultrasonography (Toshiba Aplio XV, linear probe 5–10 MHz) in all subjects at enrolment. ATX was defined at a tendon's maximum anteroposterior diameter thickness > 6.1 mm. Subjects with a history of inflammatory or degenerative tendinopathy or with previously reported Achilles tendon traumatic injury were excluded from the study.

TC, HDL-C and TG were measured using standard enzymatic methods; the LDL-C levels were calculated according to the Friedwald formula.

Hs-CRP plasma concentrations were evaluated by a commercial ELISA kit (apDia, Turnhout, Belgium, cod. 740011).

1.2. Abdominal aortic aneurysm (AAA) subjects

Subjects included in this clinical setting were provided by University Hospital, Plzen, after giving informed consent, conformed with the principles included in the Declaration of Helsinki, and approved by the Ethical Committee of the Faculty of Medicine in Plzen (12/04/2014), before to be included in the study. We tested patients with abdominal aortic aneurysm undergoing open resection or endovascular procedure-EVAR (AAA, n=30), among the patients referred to vascular surgery, and control patients, undergoing aortobifemoral bypass surgery, with atherosclerotic stenosis in the abdominal aorta or other arteries (non-AAA CTRL, n=21).

For every subject, a Computed Tomography Angiography was performed and the sample size was calculated *a priori* using the G-Power Software. Blood drawing was performed after overnight fasting. Serum aliquots were stored at −80°C and the samples were slowly defrosted in ice.

For this population, concentrations of TG, TC, and HDL-C were measured using enzymatic methods with photometric detection on Cobas system (Cobas 8000 Analyzer, Cobas c702 module, Roche Diagnostics) while LDL-C was calculated using the Friedwald equation. C-reactive protein (hs-CRP) was measured with a commercially available high-sensitivity ELISA kit (Thermo Fisher Scientifics, Italy).

1.3. Alzheimer's disease (AD) subjects

This population included seventy AD outpatients and seventy-four age- and sex-comparable cognitively normal controls (CN) without a personal or family history of neurological or psychiatric disorders recruited at the IRCCS "San Gerardo dei Tintori", Monza (Italy) and the Center E. Grossi Paoletti and the Ospedale Policlinico in Milano (Italy). All patients were seen for the first time for clinical manifestations of cognitive symptoms and were evaluated to confirm or not AD diagnosis. AD was initially diagnosed according to the NINCDS-ADRDA criteria[311] and alternative diagnoses were excluded by brain MRI scan, a routine extensive neuropsychological test battery, and evaluation of CSF biomarkers. For each AD subject the current score at the Mini-Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) was reported, while in controls was established a lack of cognitive impairment by a clinical interview, including a MMSE score > 26, CDR = 0.

For all patients, pharmacological therapy was recorded, with specific attention to lipid-modifying drugs. Patients and controls with recent infections or surgery (6 months), or under anti-infammatory, corticosteroid, or immunosuppressive drug treatments, or affected by kidney or liver failure were excluded. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and its later amendments and was approved by the internal ethical

committee (Comitato Etico Brianza, approval #3267 of May 21, 2020) and all subjects signed an informed consent.

Blood samples were collected from all patients and controls after over-night fasting and immediately centrifuged. Plasma aliquots were frozen at -80° C and slowly defrosted before use.

APOE genotype was evaluated from total DNA, extracted from peripheral blood using a commercial DNA extraction kit (Qiagen, Venlo, Netherlands), and DNA amplification was performed using specific primers as previously reported[312].

$A\beta_{1-40}$, $A\beta_{1-42}$, T-tau, and P-tau were evaluated using commercially available kits Fujirebio© using the Lumipulse G600II instrument[313]. Cut-off values for AD diagnosis were the following (normal values are reported): $A\beta_{1-42}$ > 599 pg/mL; $A\beta_{1-40}$ n.a.; T-tau < 404 pg/mL; P-tau < 56.5 pg/mL; $A\beta_{1-42}$/ $A\beta_{1-40}$ ratio > 0.069; $A\beta_{1-42}$/T-tau ratio > 1.275; $A\beta_{1-42}$/P-tau ratio > 8.1.

Serum lipid-lipoprotein profile, including total cholesterol, HDL-cholesterol, triglycerides, apoAI, apoAII, apoE, and apoB, was determined using a Roche Integra c311 autoanalyzer. LDL-C was calculated by Friedewald's formula.

1.4. Familial Hypercholesterolemia (FH) subjects in treatment with PCSK9-i

In this population were included 31 FH subjects, previously genetically confirmed for FH and all enrolled from the Lipid Centers of the "Azienda Ospedaliera Universitaria Policlinico" of Palermo, Sicily, Italy, Niguarda Hospital of Milano, Italy, and Garibaldi Hospital/University of Catania, Sicily, Italy, from January 2018 to September 2020. Partecipants age at the moment of enrollment was 18 – 70 years and LDL-C was beyond the recommended targets[11].

All the subjects were in treatment with high-intensity statins (atorvastatin 40 – 80 mg, rosuvastatin 20 – 40 mg) plus ezetimibe for at least 6 months, to reach the therapeutic target for the lipid profile parameters. Subjects taking non-statin lipid-lowering therapy were excluded from the study. Hematological and clinical evaluations were performed after a 12 h fasting, on all participants; furthermore, biochemical analyses were collected at baseline (T0) and after 6 months (T1) of PCSK9-i treatment, specificaly six subjects added alirocumab 150 mg and 25 subjects added Evolocumab 140 mg, every 2 weeks. BMI, arterial hypertension as well as smoking and glycemic status were performed in all participants at the baseline; moreover, detailed pharmacological intake as well as CV diseases history were obtained. All procedures were followed in accordance with the ethical standards of the local institutional committees on human experimentation and according to the Helsinki Declaration of 1964, as revised in 2013.

2. Cell lines

J774A.1, CHO cell lines were used in the evaluation of serum HDL-CEC, while THP-1 cell line was used in the evaluation of serum CLC.

- J774A.1: this cell line (Sigma-Aldrich, Milano, Italy), derived from macrophage/monocyte tumor cells of a female BALB/c mouse, has been grown in adherence in Dulbecco's Modified Eagle's Medium (DMEM; Euroclone, Milan, Italy) supplemented with 1 % penicillin/streptomycin (P/S; Thermo Fisher Scientific, Waltham, MA, USA), and completed with 10 % FCS (Euroclone, Milan, Italy); it was selected for HDL-CEC evaluation based on the mechanism of promoting cholesterol efflux ABCA1-mediated in presence of 0.3 mM of cyclic adenosine monophosphate (cpt-cAMP; Sigma-Aldrich, Milan, Italy) dissolved in saline solution stored in aliquots at - 20 ° C;
- CHO: Chinese hamster ovary (CHO-K1; ATCC, Manassas, VA, USA) cells are adherent cells and are used to evaluate ABCG1 transporter-mediated HDL-CEC, which is calculated by subtracting the efflux measured on the cells transfected with ABCG1 gene (CHO-L3) from the efflux measured on non-transfected cell line used as a control (CHO-K1). CHO-K1 are cultured in Ham's Nutrient Mixture F-12 (Euroclone, Milan, Italy) medium supplemented with 1 % P/S and completed with 10 % FCS, while CHO-L3, transfected with ABCG1, were maintained in Ham's F-12 medium supplemented with 1 % P/S and 0.2 % Zeocin (Thermo Fisher Scientific, Waltham, MA, USA) and completed with 10 % FCS;
- THP-1: human monocyte-derived macrophages obtained from patients with monocytic leukemia and are cells in suspension (by ECACC, purchased from Sigma-Aldrich, Milan, Italy). The cells are maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Euroclone, Milan, Italy) with HEPES supplemented with β-mercaptoethanol 50mM, gentamicin 50 mg/ml, glucose 2.5 mg/ml, NA-pyruvate 1 mM (all from Thermo Fisher Scientific, Waltham, MA, USA) and completed with 10 % FCS. This cell line is differentiated for 72 hours in the presence of 100 ng/ml forbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Milan, Italy) to generate adherent macrophages for measuring the ability of serum to promote cholesterol internalization.

Cells were kept in sterile flasks and incubated at 37 ° C with 90 – 95 % humidity and 5 % CO2. When adherent cells reached about 90 % confluence, they were washed with sterile PBS (Euroclone, Milan, Italy) and detached. Specifically, macrophages were detached mechanically with the use of a sterile scraper, while all other cell lines were detached enzymatically with trypsin in order to be seeded in plates and used in the experiments.

3. Cholesterol Efflux Capacity of HDL (HDL-CEC) assay

The ability of HDL to enhance cholesterol efflux from macrophages (HDL-CEC) was evaluated in the HDL fraction from the serum of subjects included in the study. The HDL fraction was isolated from apoB-containing lipoproteins by incubating whole serum with polyethylene glycol[314]. This technique is comparable to the isolation of HDL by ultracentrifugation for the study of cholesterol efflux capacity[315].

Sera were slowly thawed on ice prior to this procedure to prevent lipoprotein remodeling. HDL-CEC was assessed by a standardized radioisotope cell technique, through the main pathways[316].

3.1. Aqueous diffusion- and ATP-binding cassette transporter A1- mediated HDL-CEC

Aqueous diffusion (AD) and ATP-binding cassette transporter A1 (ABCA1)- mediated HDL-CEC were evaluated in a murine macrophage cell model (J774A.1, Sigma-Aldrich, Milano, Italy). Specifically, J774A.1 cells were used under basal conditions to assess AD HDL-CEC, whereas for ABCA1 HDL-CEC, J774A.1 were treated with a cyclic AMP analog (cpt-cAMP 0.3 mM; Sigma Aldrich, Milan, Italy) to induce ABCA1 gene expression[317], and the specific contribution of ABCA1 was calculated as the difference in percentage between cells treated and not treated with cpt-cAMP. J774A.1 cells were seeded in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (penicillin-streptomycin from Thermo Fisher Scientific, Waltham, MA, USA) completed with 10 % fetal calf serum (FCS) (DMEM and FCS both from Euroclone, Milan, Italy). After 24 hours, J774A.1 cells were radiolabeled with [1,2-^{3}H(N)]-Cholesterol (PerkinElmer, Waltham, MA, USA) for 24 hours at 2 µCi/ml in the presence of 2 µg/ml of an inhibitor of the cholesterol esterifying enzyme, acyl-coenzyme A: cholesterol acyltransferase (ACAT) (Sandoz 58035; Sigma-Aldrich, Milano, Italy), to prevent the formation of intracellular cholesterol esters. A cell incubation was performed for 18 hours in DMEM containing 0.2 % bovine serum albumin (BSA; Sigma-Aldrich, Milan, Italy) in presence or absence of cpt-cAMP. Cells were then incubated with 2 % (v/v) HDL serum fraction from healthy subjects undergoing the fasting program under medical supervision at the three blood sampling times, for 4 hours. HDL-CEC was expressed as the percentage of ^{3}H-cholesterol released into the medium relative to total cellular radioactivity internalized into the macrophage monolayer. Internal positive controls including lipid-free human apolipoprotein AI (human apoAI; Sigma-Aldrich, Milan, Italy) and a standard serum HDL fraction isolated from a pool of normolipidemic subjects were tested in each assay to assess proper cell reactivity. HDL-CEC values from control samples were used to minimize inter-assay variability. The intra-assay coefficients of variation (cv) for the HDL-CEC assays were < 10 %.

3.2. ATP-binding cassette transporter G1- mediated HDL-CEC

ATP-binding cassette transporter G1 (ABCG1)-mediated HDL-CEC was evaluated in Chinese Hamster Ovary (CHO-K1; ATCC, Manassas, VA, USA) cell line transfected with the human ABCG1 gene and non-transfected. The specific contribution of ABCG1 was calculated as the difference between the HDL-CEC assessed in CHO cells transfected with ABCG1 and the HDL-CEC measured in non-transfected cells. In detail, CHO cells were plated in Ham's F-12 (Euroclone, Milan, Italy) in the presence of antibiotics (penicillin-streptomycin and Zeocin from Thermo Fisher Scientific, Waltham, MA, USA), and completed with 10 % FCS. After 24 hours of labeling with [1,2-^{3}H(N)]-Cholesterol at 1 µCi/ml, CHO cells were equilibrated for 90 minutes in medium containing 0.2 % BSA. Cells were then incubated with 1 % (v/v) of the HDL serum fraction from healthy subjects undergoing the fasting program under medical supervision at the three blood sampling times, for 6 hours. HDL-CEC was expressed as the percentage of ^{3}H-cholesterol released into the medium relative to the total radioactivity of the cells internalized into the macrophage monolayer. To assess adequate cell reactivity, internal positive controls, including isolated human HDL (Sigma-Aldrich, Milan, Italy) and the HDL fraction of a standard serum isolated from a pool of normolipidemic subjects, were analyzed with the serum samples in each assay. HDL-CEC values from control samples were used to minimize inter-assay variability, which was < 10 %.

4. LCAT activity index and cholesterol esterification rate

We evaluated the serum esterified/total cholesterol ratio as an index of LCAT activity[148], in triplicate, in the AAA clinical setting. Sera were analyzed fluorometrically for total and free cholesterol, in the presence and absence of the cholesterol esters-hydrolyzing enzyme cholesterol esterase, respectively, by using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR, USA). Esterified cholesterol was then calculated.

Collaborators from E. Grossi Paoletti Center (Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy) evaluated LCAT activity in AD clinical setting as previously detailed[318]. The amount of cholesteryl esters (CEs) was calculated by subtracting unesterified cholesterol from total cholesterol and the difference was multiplied by 1.68 to have a precise estimation of the CE mass. The cholesterol esterification process was evaluated in plasma samples by measuring the cholesterol esterification rate (CER), which reflects the ability of endogenous LCAT to esterify cholesterol within endogenous lipoproteins, and LCAT activity, which reflects the ability of endogenous LCAT to esterify cholesterol within exogenous reconstituted HDL[319].

5. CETP activity

Also, serum CETP activity was measured fluorometrically, in triplicate, using the commercially available CETP Activity Assay Kit (Sigma-Aldrich, Milan, Italy), in AAA clinical setting, following corporate guidelines.

6. Serum pre-β HDL migrating particles

Serum pre-β HDL content was assessed by collaborators from E. Grossi Paoletti Center (Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy) in 10 AAA patients and in 10 control patients after separation by 2D electrophoresis. Agarose gel electrophoresis was followed by non-denaturing gradient gel electrophoresis and then followed by immunodetection against human apoAI. Serum pre-β HDL content was expressed as a percentage of total apoAI signal[320].

For AD clinical setting, HDL subclasses were evaluated by collaborators from E. Grossi Paoletti Center in plasma samples by two-dimensional electrophoresis, where agarose gel electrophoresis was followed by non-denaturing gradient gel electrophoresis and then immunoblotting to detect apoE (Calbiochem, Sigma-Aldrich, Milan, Italy) and apoAI (Calbiochem, Sigma-Aldrich, Milan, Italy). Briefly, in the first dimension, 10 µL of plasma (concentrated 10 folds using Microcon ultra (Sigma-Aldrich, Milan, Italy)) were separated by charge on a 0.5 % agarose gel (Hydragel protein(e) kit, Sebia PN4120); agarose gel strips containing the pre-separated lipoproteins were then positioned onto a home-made 3–20 % nondenaturing polyacrylamide gradient gel. Separated particles were then transferred onto a nitrocellulose membrane on which apoE-containing and apoAI-containing lipoproteins were detected with anti-apoE and anti-apoAI antibodies. The relative content of pre-β HDL was calculated by using the Bio-Rad Multi-Analyst /PC Software and expressed as a percentage of total apoAI.

7. Serum Cholesterol Loading Capacity (CLC) assay

For this assay as well, subjects' sera were slowly thawed on ice immediately prior to the analysis, to avoid the remodeling of lipoproteins[215]. The ability of serum lipoproteins to promote CLC was assessed in human monocyte-derived macrophages (THP-1; Sigma-Aldrich, Milan, Italy) using a fluorometric assay[321]. Human monocytes were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; Euroclone, Milan, Italy) medium supplemented with 1 % penicillin-streptomycin, completed with 10 % FCS. To allow THP-1 differentiation into macrophages, cells were incubated for 72 hours in the presence of 100 ng/ml forbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Milan,

Italy). The cells were then exposed to 5 % lipoprotein-poor human serum (LPDS; Sigma-Aldrich, Milan, Italy) for 24 hours and then incubated for 24 hours with 10 % (v/v) whole serum from healthy subjects undergoing the fasting program under medical supervision at the three blood sampling times. At the end of incubation, cell monolayers were lysed with 10 U/ml DNase (Sigma-Aldrich, Milan, Italy) in 1 % sodium cholate solution (Sigma-Aldrich, Milan, Italy). Intracellular cholesterol was then measured fluorimetrically using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. An aliquot of cell lysate was analyzed by bicinchoninic acid assay (BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA) to measure cell protein content. CLC of the serum was expressed as micrograms of cholesterol per milligram of protein. To verify adequate cell reactivity, internal positive controls consisting of sera from a pool of hypercholesterolemic and normolipidemic subjects were analyzed with the serum samples in each experiment at the same percentage. The relative CLC values of the control samples were used to minimize interassay variability. The intra-assay cv for the CLC assays was less than 10 %.

8. Statistical analysis

G*Power software (Düsseldorf, Germany) was used for a priori sample size calculation.

Statistical analyses were carried out using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, United States). Each sample was analyzed in triplicate. Data are reported as mean ± SD for parameters presenting a normal distribution, or as median with interquartile range (IQR) (25th to 75th percentile), for parameters with skewed distribution. The D'Agostino & Pearson normality test was performed to assess the normality of distribution. The paired and unpaired two-tailed Student’s t-test, for parameters with normal distribution, and the Wilcoxon matched-pairs signed rank test or the Mann-Whitney test for paired and unpaired analyses respectively, for parameters with skewed distribution, were used to evaluate differences between the groups.

The relationship between parameters was assessed by Pearson or Spearman linear correlation analysis for data with normal and skewed distribution, respectively, using a univariate logistic regression. Statistical significance was defined as $p < 0.05$.

RESULTS PART I:

FH WITH ATX

1. Subject characteristics

The subjects were stratified based on the absence (n = 16) or presence (n = 29) of objectively and/or ultrasonographically detectable ATX. The demographic and clinical features of the population are reported in **Table 1**. The two groups are homogenous in terms of age, sex and body mass index (BMI). Moreover, no significant differences were found in terms of high sensitive-C reactive protein (hs-CRP) serum levels. In subjects with ATX, the calculated Dutch Lipid Clinic Network Score (DCLN), the score used to diagnose "clinical" FH, was significantly higher than those without ATX ($p < 0.001$), partly attributable to familiarity, with early atherosclerotic CV found to be statistically more relevant in patients with xanthoma (51.7 % vs. 18.8 %; $p = 0.03$). The percentage of smokers was higher among subjects not presenting with ATX (50 % vs. 20.7 %; $p = 0.04$), while subjects did not differ in terms of presence of hypertension or diabetes mellitus.

Characteristics	**Xanthoma**		***p* value**
	None (n = 16)	**Present (n = 29)**	
Age — *years*	42.1 ± 18.6	46.3 ± 13.9	n.s.
Male — *n. (%)*	5 (31)	11 (38)	n.s.
BMI — *Kg/m²*	24.2 ± 5.3	25.9 ± 5.8	n.s.
hs-CRP — *µg/mL*	0.54 (1.7)	0.25 (0.94)	n.s.
DLCN score	6.4 ± 3.1	11.0 ± 5.0	< 0.001
Cardiovascular risk factors — *n. (%)*			
Smokers	8 (50)	6 (21)	0.04
Arterial Hypertension	1 (6)	3 (10)	n.s.
DM	0	0	n.s.
CV diseases	3 (19)	15 (52)	0.03
Early CVE — *n. (%)*	3 (19)	3 (10)	n.s.
Lipid profile — *mg/dL*			
TC	223.8 ± 66.8	260.4 ± 80.7	n.s.
HDL-C	61.2 ± 16.6	55.9 ± 14.0	n.s.
LDL-C	141.3 ± 57.2	180.1 ± 77.0	n.s.
TG	106.9 ± 49.0	124.5 ± 77.1	n.s.
oxLDL	56.3 ± 13.7	74.4 ± 34.3	n.s.
apoB	109.7 ± 26.3	131.5 ± 44.2	n.s.
Hypolipemic therapy users — *n. (%)*			
Statin	11 (69)	16 (55)	n.s.
Ezetimibe	4 (25)	13 (45)	n.s.

Characteristics	Xanthoma		*p* value
	None (n = 16)	Present (n = 29)	
None	5 (31)	13 (45)	n.s.

Table 1 General characteristics of the study population. BMI: body mass index; hs-CRP: serum C-reactive protein; DLCN score: Dutch Lipid Clinic Network score; DM: diabetes mellitus; CV: cardiovascular; CVE: cardiovascular events; TC: total serum cholesterol; HDL-C: serum high-density lipoproteins cholesterol; LDL-C: serum low-density lipoproteins cholesterol; TG: serum triglycerides; oxLDL: oxidized LDL; n.s.: not significant. Normally distributed continuous parameters were presented as mean ± SD, and skewed continuous parameters were expressed as the median and interquartile range (defined as 25th percentile to 75th percentile). Values are reported as (%) and number of patients with the described variable in each group.

The two groups displayed no significant differences in terms of plasma lipid profile (total cholesterol, HDL-C, LDL-C and triglycerides). Similarly, plasma levels of oxidized LDL and apolipoprotein B (apoB) did not differ between groups. The intensity and type of cholesterol-lowering therapy, mainly statins and ezetimibe, do not differ either in the two groups; only a small fraction of subjects was untreated, with equal distribution between subjects with or without ATX.

2. HDL Cholesterol Efflux Capacity (HDL-CEC)

In the patients' cohort, we first evaluated the capacity of serum HDL to promote cholesterol efflux (HDL-CEC). Results are reported in **Figure 1**. By analyzing first efflux mediated by the aqueous diffusion process (AD HDL-CEC) in macrophages, we found a trend towards a reduction in AD HDL-CEC in FH patients presenting ATX compared to patients not presenting ATX (− 8 %; p = 0.053; **Figure 1A**). Regarding the efflux mediated by the ATP-binding cassette transporter A1 (ABCA1 HDL-CEC), we found higher values in FH subjects presenting ATX compared to those without ATX (+ 18.6 %; p = 0.011; **Figure 1B**). Conversely, the presence of ATX was associated with a significantly reduced HDL efflux capacity mediated by the ATP-binding cassette transporter G1 (ABCG1 HDL-CEC) (− 11 %; p = 0.016; **Figure 1C**).

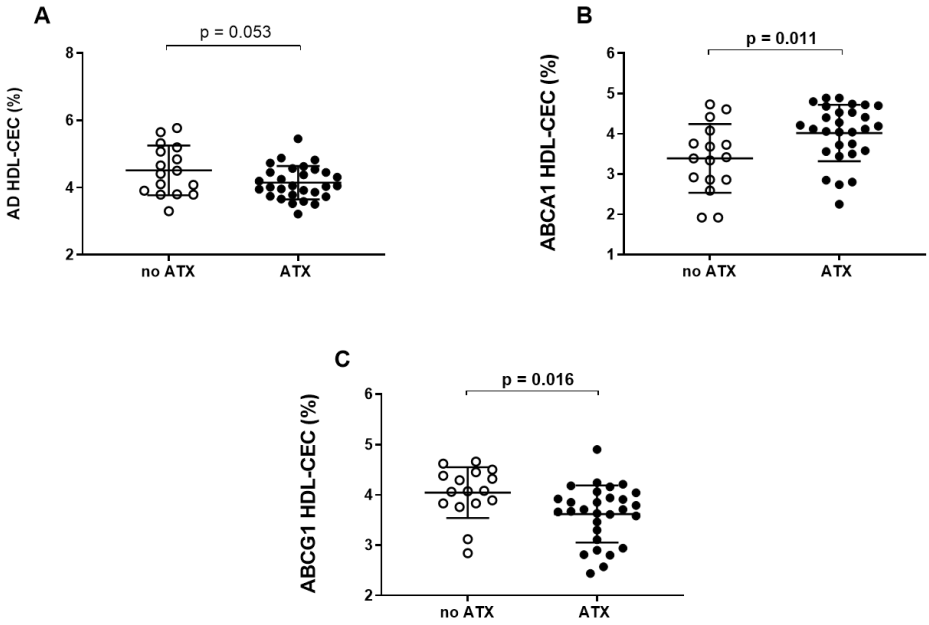

Figure 1 HDL-CEC in FH subjects not presenting (n = 16) or presenting ATX (n = 29). **(A)**: AD HDL-CEC; **(B)**: ABCA1 HDL-CEC; **(C)**: ABCG1 HDL-CEC. Each point of the graph represents the average percentage of triplicate analyses for each sample. The average of each group is represented by a horizontal, solid line. ○ No ATX: FH subjects not presenting ATX; ● ATX: FH subjects presenting ATX. Significant values are shown in bold.

3. Serum Cholesterol Loading Capacity (CLC)

We also evaluated whether the presence of ATX is associated with changes in the pro-atherogenic potential of the serum by measuring serum cholesterol loading capacity (CLC) in macrophages in this clinical setting. We found that serum CLC was significantly increased in patients with ATX compared to those without, showing an increment of 14 % (p = 0.003; **Figure 2**).

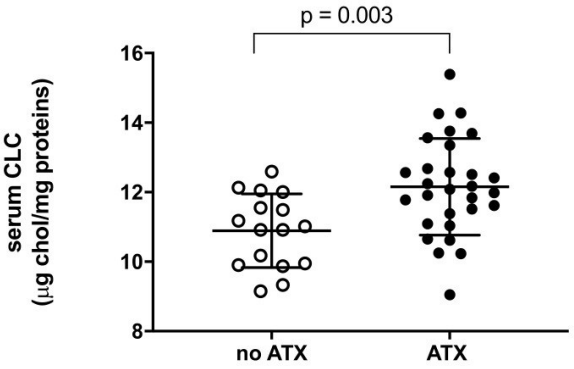

Figure 2 Serum cholesterol efflux capacity (CLC) in FH subjects not presenting or presenting ATX. Each point of the graph represents the average percentage of triplicate analyses for each sample. The average of each group is represented by a horizontal, solid line. ○ No ATX: FH subjects not presenting ATX; ● ATX: FH subjects presenting ATX.

Additionally, by exploring the relationships between serum lipoprotein function parameters, we found an inverse and robust correlation between serum CLC and the ABCG1 HDL-CEC (**Figure 3**),

while no associations were found between CLC and the other cholesterol efflux pathways (data not shown).

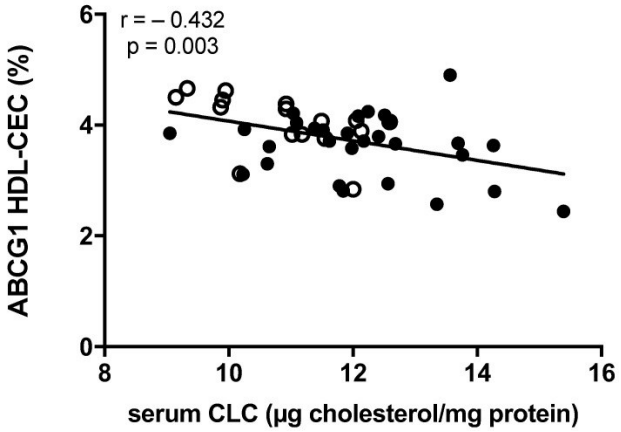

Figure 3 Correlation between ABCG1 HDL-CEC and serum cholesterol loading capacity (CLC) in FH subjects. Pearson correlation coefficient was reported. ○: subjects not presenting ATX; ●: subjects presenting ATX.

4. Correlation between Lipoprotein Functions and ATX

We then explored the possible association between serum lipoprotein functions and ATX.

Concerning the relationship of ATX with HDL-CEC through the different pathways, AD and ABCG1 HDL-CEC showed inverse and significant correlations with ATX (**Table 2**). ATX did not correlate with HDL-C levels (r = −0.255; p = 0.091).

HDL-CEC Pathways	r	*p* value
AD HDL-CEC	**– 0.342**	**0.021**
ABCA1 HDL-CEC	0.186	0.221
ABCG1 HDL-CEC	**– 0.367**	**0.013**

Table 2 Correlation between HDL-CEC and ATX. Correlation analyses were performed to highlight the relationship between parameters, and the Spearman correlation coefficients were reported. Significant associations are shown in bold.

Moreover, we observed a robust association between serum CLC and ATX (r = 0.642; $p < 0.0001$; **Figure 4**). Notably, ATX was independent of plasma LDL-C (r = 0.194; $p = 0.202$), oxLDL (r = 0.099; $p = 0.601$) and apoB plasma levels (r = 0.141; $p = 0.357$).

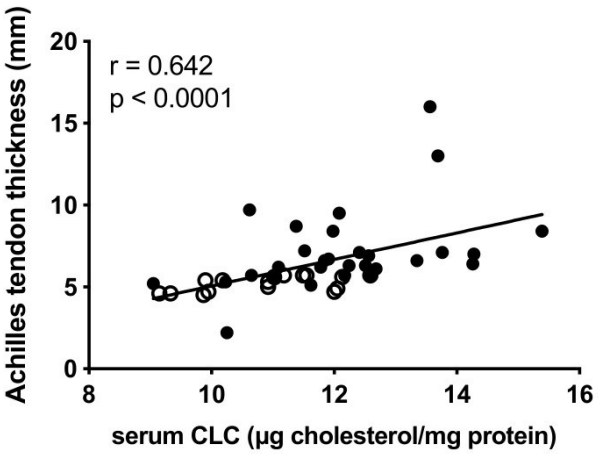

Figure 4 Correlation between serum CLC and Achilles tendon thickness in FH subjects. Spearman correlation coefficient was reported. ○: subjects not presenting ATX; ●: subjects presenting ATX.

RESULTS PART II:

AAA

1. Subject characteristics

Serum lipid profile and the other clinical parameters including hs-CRP did not differ between AAA (n = 30) and control patients (n = 21), except for age, in which it was slightly higher in the AAA group (**Table 3**).

	Control patients (*n* = 21)	**AAA patients (*n* = 30)**	***p* value**
Male — *n. (%)*	15 (71)	23 (77)	n.s.
Age — *years*	62 ± 10.14	68 ± 6.30	$p < 0.05$
BMI — *Kg/m²*	27.17 ± 3.81	26.85 ± 4.38	n.s.
Smokers — *n. (%)*	16 (76)	23 (77)	n.s.
Previous CVE — *n. (%)*	10 (48)	11 (37)	n.s.
CIHD — *n. (%)*	8 (38)	9 (30)	n.s.
LEAD — *n. (%)*	9 (43)	9 (30)	n.s.
Hypertension — *n. (%)*	14 (67)	23 (77)	n.s.
DM — *n. (%)*	5 (24)	8 (27)	n.s.
TC — *mg/dL*	151.48 ± 65.52	168.72 ± 56.16	n.s.
LDL-C — *mg/dL*	72.35 ± 44.86	67.83 ± 43.54	n.s.
HDL-C — *mg/dL*	58.72 ± 35.65	57.00 ± 42.11	n.s.
Non HDL-C — *mg/dL*	86.36 ± 56.51	91.13 ± 58.49	n.s.
TG — *mg/dL*	112.02 ± 68.85	176.14 ± 121.53	n.s.
Statin users — *n. (%)*	4 (19)	9 (30)	n.s.
hs-CRP — *µg/mL*	2.19 ± 0.57	2.09 ± 0.76	n.s.

Table 3 Clinical and biochemical parameters of control and AAA patients. Values are reported as mean ± SD or (%) and number of patients with the described variable in each group. BMI: body mass index; CVE: cardiovascular events; CIHD: chronic ischemic heart disease; LEAD: lower extremity arterial disease; DM: diabetes mellitus; TC: total serum cholesterol; LDL-C: serum low-density lipoproteins cholesterol; HDL-C: serum high-density lipoproteins cholesterol; TG: serum triglycerides; hs-CRP: serum C-reactive protein. n.s.: not significant.

2. HDL cholesterol efflux capacity (HDL-CEC)

ABCG1 HDL-CEC in AAA was 16 % lower than in control patients ($p < 0.001$) and with more dispersed values (**Figure 5A**). On the contrary, ABCA1 HDL-CEC was significantly higher in patients with AAA (+ 31.7 %, $p < 0.0001$) (**Figure 5B**). AD HDL-CEC showed a non-statistically significant trend towards a reduction in AAA (**Figure 5C**).

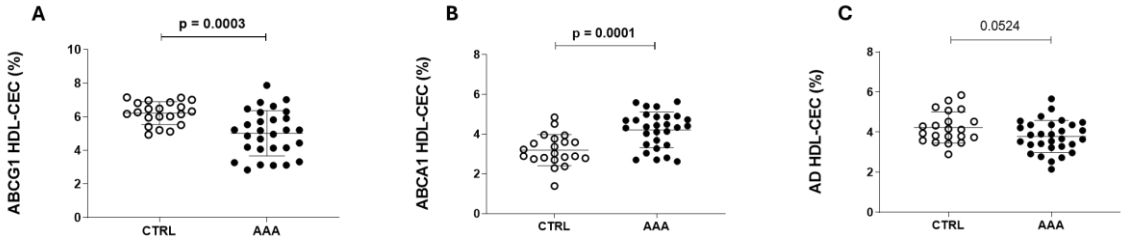

Figure 5 HDL-CEC in AAA (n = 30) and non-AAA control patients (n = 21). Panel **(A)** refers to ABCG1 HDL-CEC; panel **(B)** refers to ABCA1 HDL-CEC; panel **(C)** refers to AD HDL-CEC. The mean and SD for each group are reported. Unpaired two-tailed Student's t-test for parameters with normal distribution was used. ○: control subjects; ●: subjects with AAA. Significant values are shown in bold.

3. LCAT and CETP activity

As HDL-CEC depends on the different subpopulations of HDL formed as a result of the action of remodeling enzymes, such as LCAT, and lipid transfer proteins, such as CETP, LCAT and CETP activity levels were assessed in this clinical setting. LCAT activity, measured indirectly through the evaluation of the serum esterified/total cholesterol ratio, was significantly higher in AAA than in control patients (+ 23 %, $p < 0.0001$) (**Figure 6A**).

CETP activity was significantly higher in AAA than in control patients (+ 49 %, $p < 0.0001$) (**Figure 6B**). A direct significant relationship between LCAT and CETP activity ($R^2 = 0.143$, $p < 0.05$) was found only in the AAA group (**Figure 6C**).

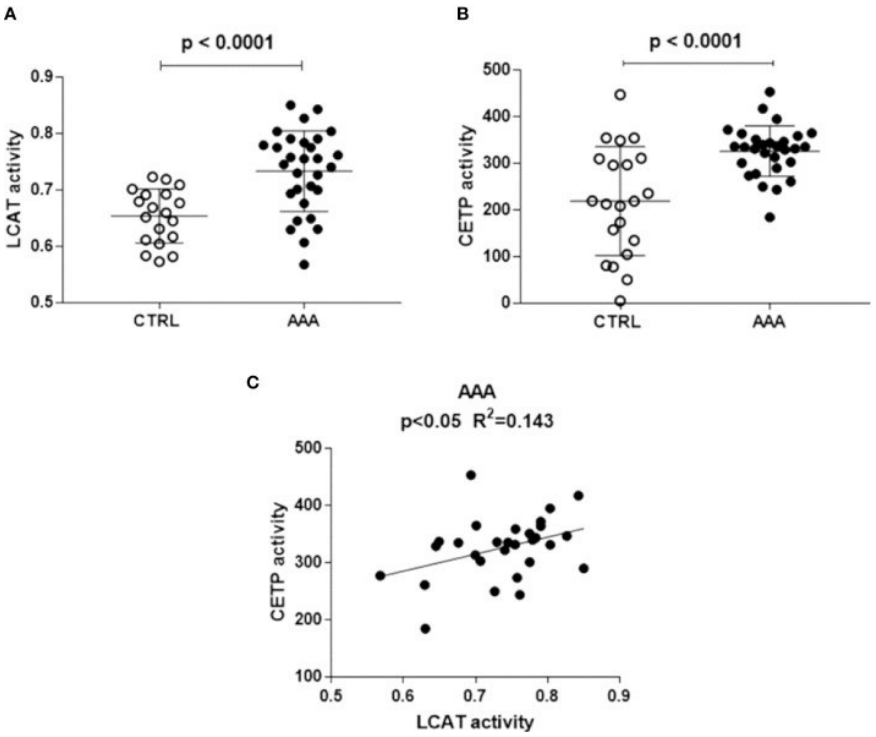

Figure 6 LCAT and CETP activity in AAA and non-AAA control patients. LCAT and CETP activity is shown in panels **(A, B)**. Mean and SD for each group are reported. Unpaired two-tailed Student's t-test for parameters with normal distribution was used. The significant relationship between LCAT and CETP activity, found only in AAA patients by linear regression analysis, is shown in panel **(C)**. ○: control subjects; ●: subjects with AAA.

4. Correlation of HDL-CEC with other parameters

None of the efflux pathways correlated with other parameters in the control group of patients (data not shown). Conversely, in the AAA group, ABCG1 HDL-CEC correlated inversely with ABCA1 HDL-CEC ($R = -0.365$, $p < 0.05$) (**Figure 7A**) and directly with AD HDL-CEC ($R = 0.676$, $p < 0.0001$) (**Figure 7B**). Moreover, we found an inverse correlation between ABCA1 HDL-CEC and serum HDL levels in the same group ($R = -0.422$; $p < 0.05$) (**Figure 7C**).

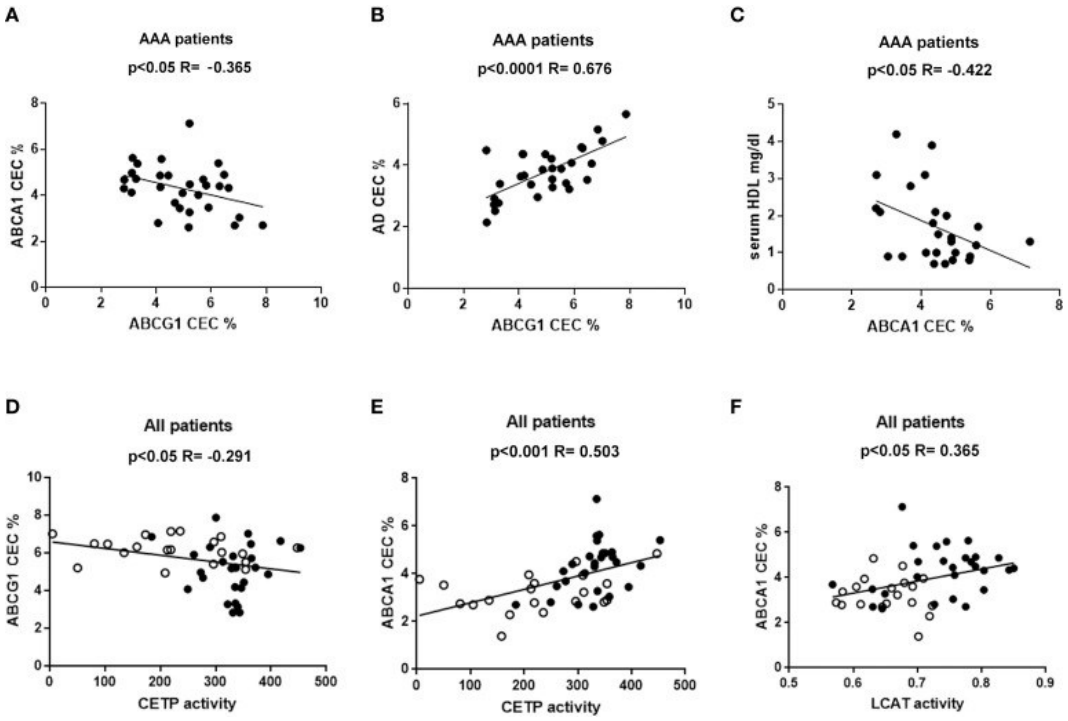

Figure 7 Correlations between HDL CEC and other parameters. Panels **(A–C)** refer to statistically significant correlations found only in AAA patients. Panels **(D–F)** show the statistically significant correlation found considering together non-AAA control (o) and AAA patients (●).

Considering all patients together, ABCG1 HDL-CEC and CETP activity were inversely correlated ($R = -0.291$, $p < 0.05$) (**Figure 7D**), while ABCA1 HDL-CEC was directly correlated both with LCAT and CETP activity ($R = 0.503$, $p < 0.001$, and $R = 0.365$, $p < 0.05$, respectively) (**Figures 7E, F**). ABCA1 HDL-CEC was significantly directly correlated with CETP, also considering AAA patients separately ($R = 0.434$, $p < 0.05$).

5. Effect of smoking habit on HDL-CEC and activity of LCAT and CETP

Since a strong risk factor for AAA is cigarette smoking[322], we evaluated the effect of smoking habit on the serum functional parameters. We selected patients who were smokers or non-smokers at the time of the study, excluding past smokers, in the two groups. In AAA, ABCG1 HDL-CEC was significantly lower and ABCA1 HDL-CEC was significantly higher in smokers than in non-smokers (−22.2 %, $p < 0.05$, + 27.5, $p < 0.05$, respectively) (**Figures 8A, B**), with no difference in serum lipid profile (data not shown). We found no difference between smokers and non-smokers within the control patient group (**Figures 8C, D**).

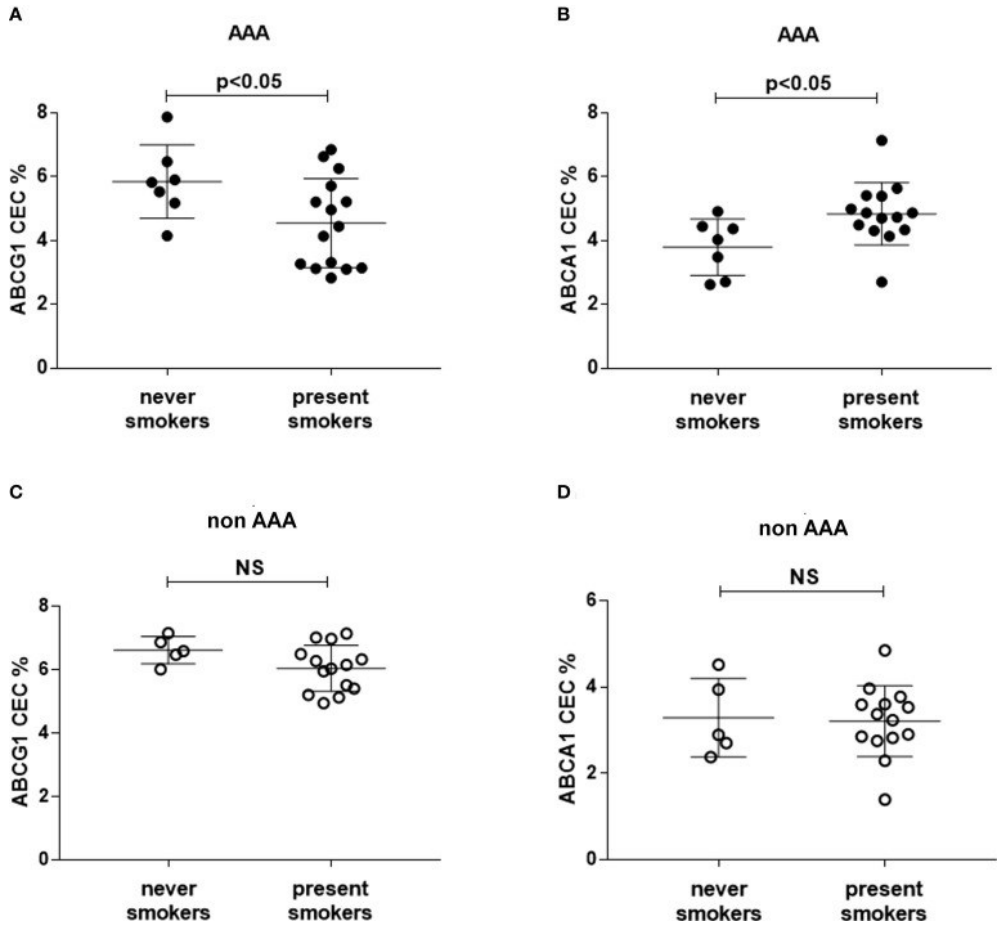

Figure 8 HDL-CEC in smoker and non-smoker patients. ABCG1 HDL-CEC **(A, C)** and ABCA1 HDL-CEC **(B, D)** values were stratified into never (n=5 and n=7 for controls and AAA, respectively) and present smokers (n=14 and n=15 for controls and AAA, respectively). Mean and SD for each group are reported. The unpaired Mann–Whitney test for statistical analysis of parameters with skewed distribution was applied. NS, not significant.

Stratification by smoking did not produce significant differences in LCAT and CETP activity (data not shown).

6. Serum pre-β HDL migrating particles

Given the previously reported role of these particles in promoting cholesterol efflux, mostly by interacting specifically with ABCA1[323,324] and to a limited extent with ABCG1[325], we evaluated serum pre-β HDL content in this clinical setting. The measurement of serum pre-β HDL particles as percentage over total apoAI signal showed significantly lower levels in AAA patients (19.1 ± 5.5 %) than in control patients (10.4 ± 10.3 %) (**Figure 9**).

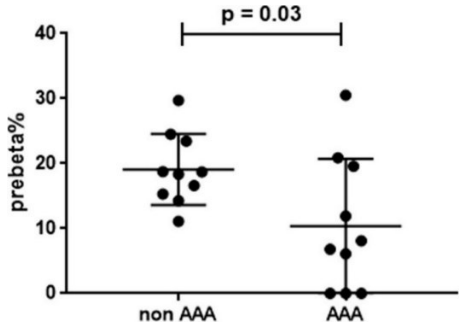

Figure 9 Serum pre-β HDL particles in AAA and non-AAA control patients. The mean and SD for each group are reported. The unpaired two-tailed Student's t-test was applied.

7. Serum cholesterol loading capacity (CLC)

CLC was not different between the two groups of patients (**Figure 10**), and between smokers and non-smokers within each group (data not shown).

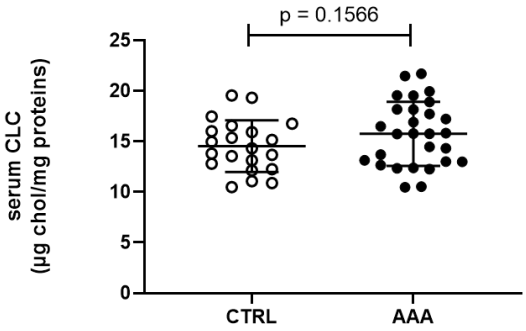

Figure 10 Serum CLC in AAA and non-AAA control patients. The mean and SD for each group are reported. Unpaired two-tailed Student's t-test for parameters with normal distribution was used. ○: control subjects; ●: subjects with AAA.

8. Correlation of serum CLC with other parameters

No correlation was found between CLC and other parameters in the control group (data not shown). Conversely, in the AAA group, CLC correlated directly with ABCG1 HDL-CEC ($R = 0.463$, $p < 0.05$) and with AD HDL-CEC ($R = 0.458$, $p < 0.05$) (**Figures 11A, B**). An inverse correlation was found between CLC and ABCA1 HDL-CEC ($R = -0.484$, $p < 0.01$), and with CETP activity ($R = -0.490$, $p < 0.01$) (**Figures 11C, D**).

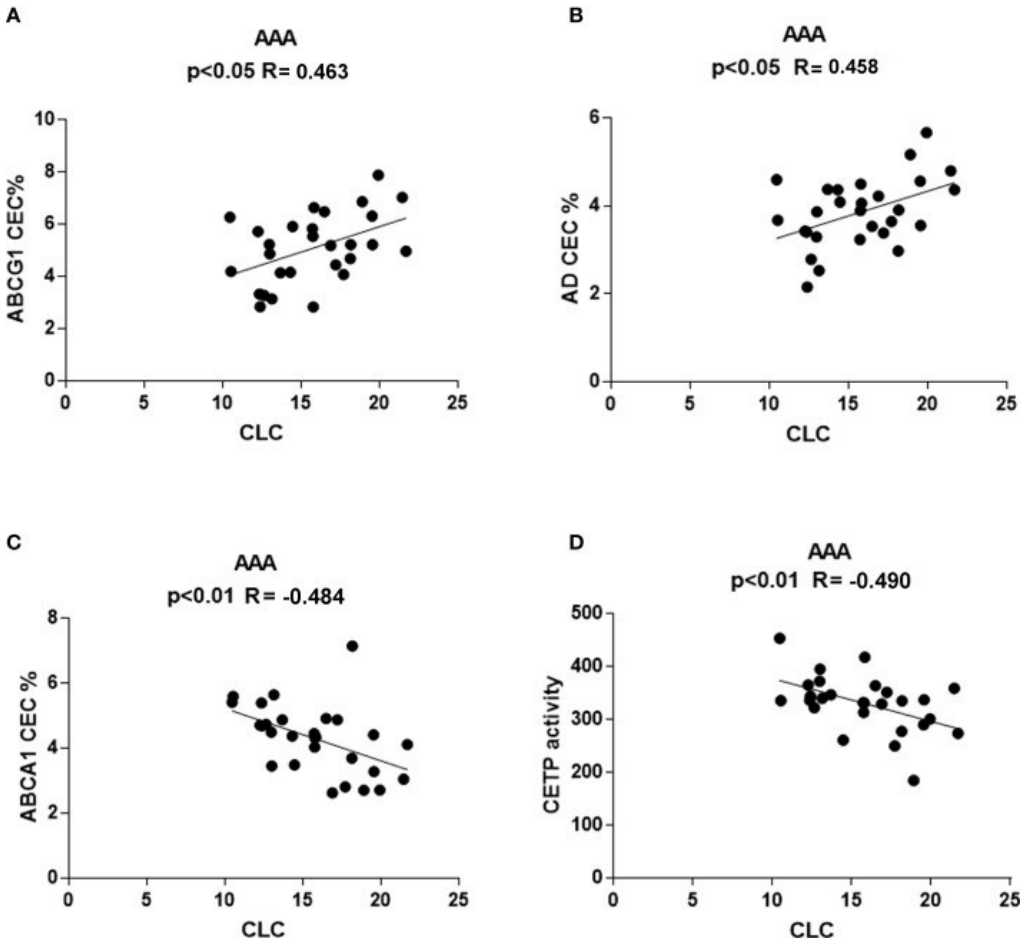

Figure 11 Correlations between serum CLC and other parameters in AAA patients. Positive correlation of CLC with ABCG1 HDL-CEC **(A)** and AD HDL-CEC **(B)** and inverse correlation of CLC with ABCA1 HDL-CEC and CETP **(C)** activity **(D)** were found only in AAA patients.

RESULTS PART III:

AD

1. Subject characteristics

The characteristics of the enrolled AD patients (n = 70) and cognitively normal controls (n = 74) are reported in **Table 4**. The two groups were comparable for age and sex distribution. AD patients had an average MMSE of 20.9 and CDR of 1.4, in line with initial clinical manifestations. Liquor markers were per protocol confirmatory of AD in all the enrolled patients. APOE genotype distribution in the AD group was different from that of the control group and characterized by a predominance of carriers of the APOEε4 allele, as described in previous Italian AD cohorts[326,327]. More than one-fourth of AD patients were taking statins, while only 6.8 % of controls were treated.

	AD	**CN**
N	70	74
Sex — *M/F*	34/36	37/37
Age — *years*	73.6 ± 6.6	70.8 ± 9.8
MMSE score	20.9 ± 4.2	
CDR score	1.4 ± 0.7	
Disease biomarkers[a]		
$A\beta_{1-42}$ — *pg/mL*	442 (303; 572)	1,034 (608; 1,765)
$A\beta_{1-40}$ — *pg/mL*	10,482 (7838; 12,869)	n.a.
T-tau — *pg/mL*	673 (464; 965)	176 (93; 325)
P-tau — *pg/mL*	100 (78; 147)	33 (24; 48)
$A\beta_{1-42}/A\beta_{1-40}$	0.044 (0.038; 0.051)	n.a.
$A\beta_{1-42}$/T-tau	0.67 (0.36; 1.09)	6.40 (2.01; 14.53)
$A\beta_{1-42}$/P-tau	4.4 (2.5; 6.3)	30.34 (13.78; 53.13)
Apolipoprotein E genotype — *n. (%)*[b]		
E4/E4	4 (6)	2 (6)
E3/E4	30 (44)	4 (12)
E3/E3	33 (48)	25 (74)
E2/E3	2 (3)	3 (9)
Statin users — *n. (%)*	19 (27)	5 (7)

Table 4 Demographic and clinical data of Alzheimer's disease patients and control subjects. AD: Alzheimer's disease, CN: cognitive normal controls, MMSE: Mini-Mental State Examination, CDR: Clinical Dementia Rating Scale
Normally distributed continuous parameters were presented as mean ± SD, and skewed continuous parameters were expressed as the median and interquartile range (defined as 25th percentile to 75th percentile). Values are reported as (%) and number of patients with the described variable in each group.
[a]Disease biomarkers were available for 21 controls
[b]Apolipoprotein E genotype was evaluated in 69 AD patients and in 34 controls

2. Plasma lipid/lipoprotein profile in AD patients

Plasma lipid and apolipoprotein levels were comparable between AD patients and controls, except for plasma unesterified cholesterol concentration, which was significantly lower in AD patients, thus leading to a strongly reduced unesterified/total cholesterol ratio (**Table 5**). Differently from previously reported data[287], plasma HDL-C, apoAI, and apoAII levels were in the normal range in AD patients. Plasma HDL subclass distribution in AD patients was comparable to controls (**Table 5**), but the content of small discoidal pre-β HDL particles was reduced in AD patients (**Table 5**).

	AD (*n* = 70)	**CN (*n* = 74)**	***p* value**
TC — *mg/dL*	193.7 ± 44.3	198.4 ± 39.7	0.64
Unesterified-C — *mg/dL*	44.0 ± 15.7	56.0 ± 9.8	**< 0.0001**
Unesterified/TC	0.23 ± 0.08	0.28 ± 0.03	**0.0002**
CE — *mg/dL*	251.3 ± 70.1	244.9 ± 54.4	0.66
LDL-C — *mg/dL*	117.9 ± 38.6	106.9 ± 32.1	**0.06**
HDL-C — *mg/dL*	54.0 ± 16.1	57.1 ± 12.1	0.11
Non-HDL-C — *mg/dL*	139.7 ± 40.9	137.9 ± 36.3	0.78
TG — *mg/dL*	105.0 (82; 129)	106.5 (75; 123)	0.95
Phospholipids — *mg/dL*	209.2 ± 39.8	212.3 ± 42.1	0.94
apoAI — *mg/dL*	143.2 ± 27.8	138.8 ± 20.9	0.29
apoAII — *mg/dL*	28.6 ± 4.7	29.1 ± 7.3	0.83
apoE — *mg/dL*	3.4 ± 1.1	2.9 ± 0.8	0.37
apoB — *mg/dL*	96.7 ± 24	110.9 ± 31.4	0.03
LCAT activity — *nmol/mL/h*	29.4 ± 12.4	36.4 ± 7.8	**< 0.0001**
CER — *nmol/mL/h*	30.2 ± 12.9	35.9 ± 12.0	0.02

	AD (*n* = 70)	CN (*n* = 74)	*p* value
Pre-β HDL — *(%) of total apoAI*	9.95 ± 6.40	13.63 ± 4.12	0.03

Table 5 Plasma lipid/lipoprotein profile of Alzheimer's disease patients and control subjects. AD: Alzheimer's disease; CN: cognitive normal controls; TC: total serum cholesterol; CE: cholesteryl esters; HDL-C: serum high-density lipoproteins cholesterol; LDL-C: serum low-density lipoproteins cholesterol; TG: serum triglycerides; LCAT: lecithin:cholesterol acyltransferase, CER: cholesterol esterification rate.
Data are reported as mean ± SD or median (defined as 25th percentile to 75th percentile). AD and controls were compared by the Wilcoxon rank-sum test. Values in bold indicate statistically significant results.

Plasma HDL-CEC was also evaluated in this cohort of subjects (**Figure 12**). AD HDL-CEC of plasma was higher in AD patients than in controls (+ 26.4 %, p < 0.0001, **Figure 12A**), but ABCA1 HDL-CEC pathway was reduced in AD patients (- 15.9 %, p < 0.0001; **Figure 12B**), in agreement with the reduced content of discoidal pre-β HDL particles (**Table 5**), the major cholesterol acceptor via this pathway. Also, ABCG1 HDL-CEC was reduced in AD patients compared to CN controls (– 15.8 %, p < 0.0001; **Figure 12C**) despite no difference in terms of HDL-C levels.

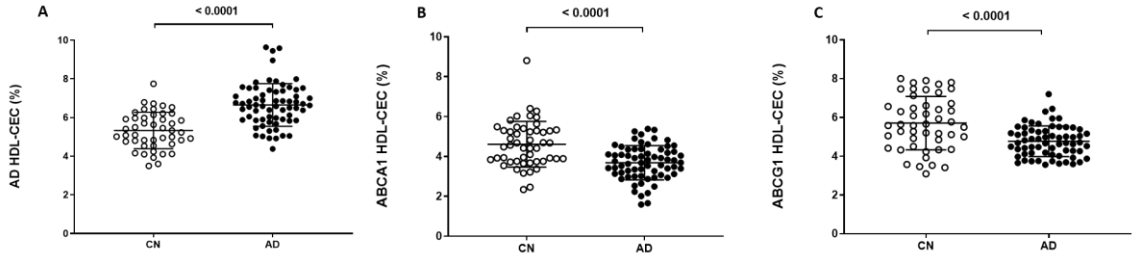

Figure 12 HDL-CEC in AD subjects (n = 70) compared to CN subjects (n = 74). **(A)**: AD HDL-CEC; **(B)**: ABCA1 HDL-CEC; **(C)**: ABCG1 HDL-CEC. Each point of the graph represents the average percentage of triplicate analyses for each sample. The mean and SD for each group are reported. Unpaired two-tailed Student's t-test for parameters with normal distribution was used. ○ CN: cognitively normal controls; ● AD: Alzheimer's Disease subjects. Significant values are shown in bold.

The evaluation of the plasma cholesterol esterification process revealed a partly compromised efficiency of the system in AD patients; LCAT activity and cholesterol esterification rate (CER) were reduced by 29 % and 16 %, respectively, in AD patients (**Table 5**). These results are in line with the strongly reduced plasma levels of unesterified cholesterol, the LCAT substrate.

Adjustment for statin use minimally affected the comparisons between plasma lipids in AD patients and controls, except for a shift to borderline significance for LDL-C (from p = 0.06 to p = 0.04) and an opposite shift for CER (from p = 0.02 to p = 0.12).

RESULTS PART IV:

FH IN TREATMENT WITH PCSK9-I

1. Subject characteristics

31 FH subjects participated at the study. In agreement with the 2019 ESC/EAS guidelines for the management of dyslipidemias and the national PCSK9-i prescriptive regulation[11,328], PCSK9-i treatment was started in all these FH subjects; specifically, six subjects added Alirocumab 150 mg and 25 subjects added Evolocumab 140 mg, every 2 weeks.

Table 6 shows the characteristics of the study population. The population was homogeneous in terms of sex, age and BMI, while more than half of patients had a previous CV event. In particular, the majority of patients suffered from coronary artery disease while cerebrovascular disease was present in 11.8 % of population; finally, 17.6 % of patients were affected by peripheral artery disease. All FH patients had heterozygous LDLR genetic variants and the most prevalent mutation class was amino acid change. Finally, the majority of FH patients were on rosuvastatin 20 mg, while the percentage of patients on atorvastatin 40 mg was 41.9 % as well as the prevalence of assumed antihypertensive treatment.

Demographic Characteristics	
N	31
Age — *years ± SD*	57 ± 11
Male — *n. (%)*	16 (52)
BMI — *Kg/m²*	25 ± 3
Smokers — *n. (%)*	6 (19)
DM — *n. (%)*	1 (4)
CV diseases — *n. (%)*	17 (55)
- Coronary artery disease, *n.* (%)	12 (71)
- Cerebrovascular disease, *n.* (%)	2 (12)
- Peripheral artery disease, *n.* (%)	3 (18)
Hypertension — *n. (%)*	13 (42)
Mutation class	
Amino acid change — *n. (%)*	17 (55)
Null allele — *n. (%)*	14 (45)
FH phenotype	

Demographic Characteristics	
Heterozygous FH — *n. (%)*	31 (100)
Treatment	
Antihypertensive therapy — *n. (%)*	13 (42)
Antiplatelet therapy — *n. (%)*	17 (55)
High-intensity statin therapy users	
Atorvastatin 40 mg — *n. (%)*	13 (42)
Rosuvastatin 20 mg — *n. (%)*	18 (58)
Ezetimibe — *n. (%)*	31 (100)

Table 6 Characteristics of the study population.BMI: body mass index; DM: diabetes mellitus; CV: cardiovascular; FH: familial hypercholesterolemia; LDLR: low-density lipoprotein receptor; PCSK9-i: proprotein convertase subtilisin/kexin type 9 inhibitors; SD: standard deviation. Data are presented as mean ± SD and (%) and number of patients with the described variable in the group.

As concerns the lipid profile, we observed a significative reduction of total cholesterol (− 41.6 %, $p < 0.0001$), and LDL-C (− 56.7 %, $p < 0.0001$) after 6 months of PCSK9-i treatment; specifically, more than half of patients achieved the recommended LDL-C target according to the recent guidelines[11]. No changes in HDL-C levels were observed (p = 0.4965). A trend to a reduction in triglyceride levels occurred, however, without reaching statistical significance (p = 0.0706) (**Table 7**). In two secondary analyses, after stratification of FH patients according to smoking and hypertension respectively, similar results were obtained after 6 months of PCSK9-i treatment (data not shown).

Lipid profile— *mg/dL*	**Before treatment HeFH (*n* = 31)**	**After 6-months PCSK9-i treatment HeFH (*n* = 31)**	***p* value**
TC	238 (205; 287)	139 (113; 181)	**<0.0001**
LDL-C	140 (109; 188)	61 (42; 110)	**<0.0001**
- LDL-C target — n. (%)	-	*16 (52)*	-
HDL-C	53 ± 11	53 ± 9	0.4965
TG	112 (74; 130)	95 (72; 118)	0.0706

Table 7 Lipid profile characteristics of study population at baseline and after 6 months of PCSK9-i treatment. HDL-C: high-density lipoproteins; HeFH: heterozygous familial hypercholesterolemia; LDL-C: low-density lipoproteins; PCSK9-i: proprotein convertase subtilisin/kexin type 9 inhibitors; TC: total cholesterol; TG: triglyceride. Data are presented as

mean ± SD, or median and interquartile range (defined as 25th percentile to 75th percentile) according to normal or skewed distribution respectively, and (%) and number of patients with the described variable in the group. Values in bold indicate statistically significant results.

No cardiovascular events occurred during 6 months of PCSK9-i treatment.

2. HDL cholesterol efflux capacity (HDL-CEC)

On the FH patient cohort, we first evaluated the capacity of serum HDL to promote cholesterol efflux (HDL-CEC) (**Figure 13**). We found a significant increase of AD HDL-CEC (+7.8 %; p = 0.0008; **Figure 13A**); however, PCSK9-i treatment promoted a trend towards a reduction of ABCA1 HDL-CEC, without reaching statistical significance (p = 0.0513) (**Figure 13B**). Treatment with PCSK9-i markedly increased ABCG1 HDL-CEC (+ 22.2 %; p < 0.0001; **Figure 13D**). In two secondary analyses, we stratified FH patients according to smoking and hypertension, respectively. We found that non-smokers as well as non-hypertensive patients exhibited a significant increase of the ABCG1 HDL-CEC after 6 months of PCSK9-i treatment (data not shown). Interestingly, by comparing HDL-CEC of treated FH patients with that of a small group of healthy control subjects added in each experiment as internal standards, we observed similar values (AD HDL-CEC of controls: 5.9 % ± 0.7, AD HDL-CEC of FH after treatment 6.0 % ± 1.2, p > 0.999; ABCG1 HDL-CEC of controls: 5.2 % ± 1.7, ABCG1 HDL-CEC of FH after treatment 5.6 % ± 1.4, p > 0.9999; ABCA1 HDL-CEC of controls: 3.6 % ± 0.6; ABCA1 HDL-CEC of FH after treatment 3.7 % ± 0.9, p > 0.9999).

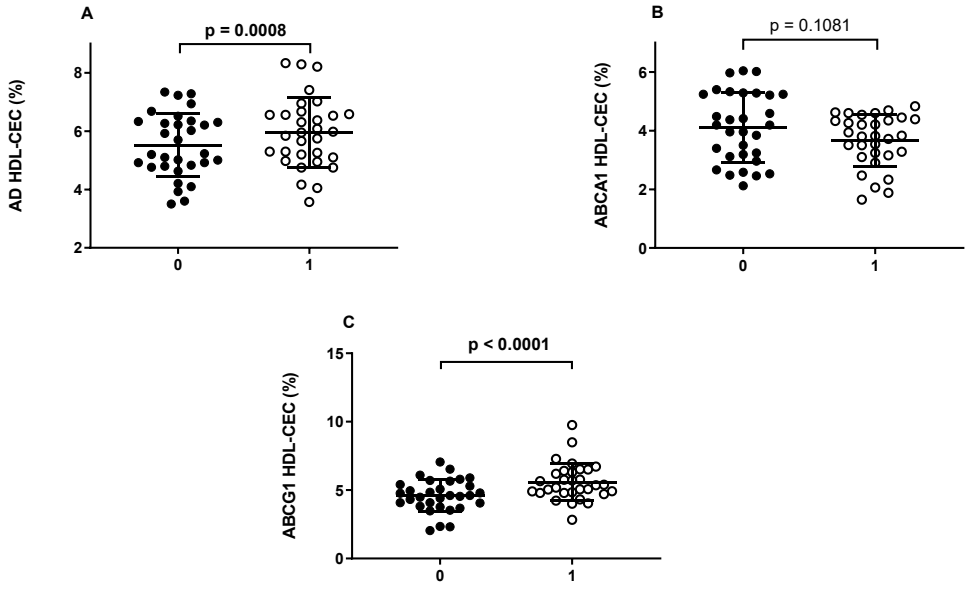

Figure 13 HDL-CEC in FH subjects (n = 31) before and after PCSK9-i treatment. **(A)**: AD HDL-CEC; **(B)**: ABCA1 HDL-CEC; **(C)**: ABCG1 HDL-CEC. Every dot of the graphs represents the mean percentage of each triplicate analysis for serum sample. The mean and SD for each group are reported. Paired two-tailed Student's t-test for parameters with normal distribution

was used. 0: FH subjects before PCSK9-i treatment; 1: FH subjects after 6 months of PCSK9-i treatment. Values in bold indicate statistically significant results.

3. Serum cholesterol loading capacity (CLC)

We also evaluated whether PCSK9-i treatment is associated to changes in the pro-atherogenic potential of the serum by measuring serum cholesterol loading capacity (CLC) in macrophages also in this clinical setting. We found that serum CLC was significantly decreased in FH subjects after 6 months of PCSK9-i treatment (− 6.6 %, $p = 0.0272$**; Figure 14**). Additionally, we found a direct but weak correlation between serum CLC and LDL-C serum levels ($R^2 = 0.091$; $p = 0.006$). In two secondary analyses, after stratification of FH patients according to smoking and hypertension respectively, we observed a significant reduction of serum CLC in smokers and hypertensive patients after 6 months of PCSK9-i treatment (data not shown). As for HDL-CEC, CLC values of sera from FH patients after PCSk9-i treatment were similar to those of a small group of healthy control added in each experiment as internal standards (CLC of control sera: 11.6 ± 1.7; CLC of FH after treatment: 14.5 ± 5.1; p = 0.4158).

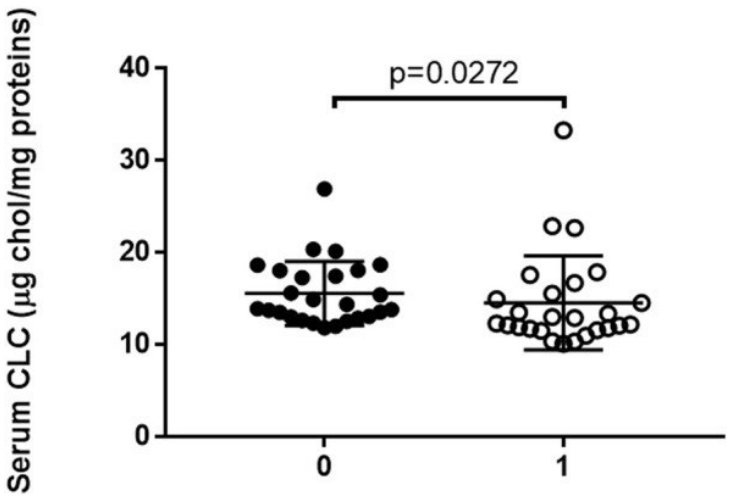

Figure 14 Serum CLC in FH subjects (N = 26) before and after 6 months of PCSK9-i treatment. Every dot of the graph represents the mean percentage of each triplicate analysis for serum sample. The mean and SD for each group are reported. Paired two-tailed Student's t-test for parameters with normal distribution was used. 0: FH subjects before PCSK9-i treatment; 1: FH subjects after 6 months of PCSK9-i treatment. 5 samples over 31 were not available for CLC determination.

DISCUSSION

The subjects included in this study are affected by Familial Hypercholesterolaemia (FH) and Abdominal Aortic Aneurysm (AAA), two diseases characterized by a high CV risk, and subjects with Alzheimer's disease (AD), included in the study because AD and CV diseases share cumulative risk factors. Among the risk factors that link these diseases, dyslipidaemia is the factor of greatest interest for this thesis. In this regard, several studies have reported a higher frequency of CVD in FH with xanthomas than in FH without xanthomas[54], while CV risk is reduced in FH subjects on PCSK9 inhibitors (PCSK9-i) proportionally to the reduction in LDL-C levels[329]. As mentioned above, AAA could be classed as a CV diseases and shares risk factors with other atherosclerotic CV diseases, although the pathophysiology of this disease is still not fully understood[330], while CV diseases and AD share several risk factors, including advanced age and altered lipid profile levels, particularly hypercholesterolemia, which may also play a role in the development of AD[99,114].

In this study, the serum lipoprotein functions (i.e., the capacity of HDL to promote cell cholesterol efflux (HDL-CEC) and serum capacity to promote cell cholesterol loading (CLC)) were evaluated in the three clinical settings, FH, AAA and AD subjects, to evaluate if disturbance of these parameters occurs and correlate with the main risk factors for these diseases and may therefore be responsible for a more severe clinical picture associated with these individuals. Additionally in this study was assessed whether a pharmacological modulation can positively affect lipoprotein function and leads to a reduction in CV risk.

Discussion Part I

As mentioned above, tendon xanthomas consist of accumulations of collagen and cholesteryl ester-containing macrophages in tendons[331], and their presence is a pathognomonic sign of FH, an inherited metabolic disorder characterized by high levels of LDL-C that predispose to the development of early CV diseases[11,52]. Achilles tendon xanthoma (ATX) plays an important role in the diagnostic definition of FH, leading the patient to early treatment of the disease, and its evaluation may provide a better CV risk assessment as it is associated with premature CV diseases[53,55].

In this clinical setting, the two groups of patients, FH-presenting ATX and FH non-presenting ATX, were homogeneous in terms of age, BMI, lipid profile and lipid-lowering treatments, variables that may influence xanthoma development[251,332].

Although higher levels of total cholesterol and LDL-C have been reported in patients with xanthoma than in those without[54], in our study, LDL-C, apoB, and oxidized LDL levels were similar in the two groups of patients. Consistent with our data, other studies reported the absence of changes in lipid levels in subjects with xanthoma[333,334]. This observation suggests that the overall ability of serum to deliver cholesterol to cells (CLC), and not just LDL-C concentrations, is relevant for ATX formation.

This is consistent with the observation that not all FH subjects develop ATX despite having high LDL-C levels[335] and sharing the same LDL receptor gene mutation[336].

Our results clearly suggest that the presence of a high serum CLC may directly contribute to cholesterol accumulation within the tendons and promote tendon thickening. This relationship is strengthened by the positive correlation between serum CLC and Achilles tendon thickness found in this clinical setting, again clearly indicating that higher serum CLC is associated with cholesterol accumulation in peripheral tissues such as macrophages in Achilles tendons. On the other hand, LDL-C, apoB and oxidative LDL do not seem to contribute to Achilles tendon thickness. In this regard, the role of a specific subclass of LDL could be speculated. In particular, the small dense LDL particles have an increased ability to induce macrophage cholesterol accumulation[337,338]. In a case report, Mancuso and coworkers found that a patient who had ATX had small and dense very-low-density LDLs (VLDLs) and LDLs. In this patient, an unusual amount of conjugated dienes of arachidonic acid was reported in the plasma and LDLs, which is present only in small traces in the control population[334]. These data suggest that in patients with xanthoma, qualitative lipoprotein abnormalities, such as an increased presence of more atherogenic LDL as the already mentioned oxidated and small dense LDL particles, may explain the higher CLC we observed.

The pathophysiological process responsible for the formation of tendon xanthomas has significant similarities with the pathogenesis and progression of atherosclerotic plaque, involving cholesterol accumulation in arterial wall macrophages and foam cell formation[248]. Thus, the direct relationship between serum CLC and Achilles tendon thickness, which reflects an association with cholesterol accumulation in macrophages, may very likely also indicate a correlation with cholesterol accumulation in the atherosclerotic plaque of the arterial wall. Consistent with this conclusion, both the presence of xanthomas and high serum CLC are associated with increased cardiovascular risk[54,215].

In addition, the higher intracellular cholesterol content of macrophages exposed to sera from ATX patients in our model may also be related to some specific inflammatory factors present in the serum that can directly modulate cellular cholesterol content[178,339]. Indeed, an increased concentration of tryptase, TNF-α, IL-8 and IL-6 in plasma of FH subjects with ATX compared to subjects without ATX has been previously reported[253]. However, in agreement with Nielsen et al[340], the presence of xanthoma was not associated with significant changes in hs-CRP levels in our cohort of patients, excluding a possible role of plasma inflammatory factors in the observed elevated CLC.

Another important factor influencing macrophage cholesterol homeostasis is the ability of HDL to interact with the membrane transporter ATP-binding cassette A1 (ABCA1) and G1 (ABCG1), thereby promoting cholesterol efflux and opposing cholesterol loading. In our study, ABCG1-mediated HDL-CEC was lower in patients with ATX and inversely correlated with CLC in the entire study population.

In addition, ABCG1-mediated HDL-CEC was inversely correlated with Achilles tendon thickness. These results suggest that the increased CLC in ATX patients and its correlation with ATX formation may be explained, at least in part, by reduced ABCG1-mediated HDL-CEC, according to the notion that serum CLC is the net result of all serum lipoprotein contributions. On the contrary, ABCA1-mediated HDL-CEC was increased in patients with ATX compared to FH patients without ATX and did not correlate with CLC, suggesting its minor role in CLC level and xanthoma formation.

The observed reduction of ABCG1-mediated HDL-CEC in ATX-presenting patients occurred despite no significant changes in serum HDL levels, consistent with the previously reported weak or absent relationship of this parameter with serum HDL-C levels[341,342]. Conversely, the ability of HDL to interact with specific membrane cholesterol transporters depends on the maturation process they undergo in serum, which generates different subclasses of HDL particles. The significantly lower ABCG1 HDL-CEC and higher ABCA1 HDL-CEC found in patients with ATX suggest a shift in HDL particle distribution towards lipid-poor pre-β HDL, which specifically interacts with ABCA1[128], with a consequent reduction in mature particles, that have a greater affinity for the ABCG1-mediated pathway[343]. Indeed, reduced HDL maturation has been observed in high-risk individuals[341].

The hypothesis of a defect in the HDL maturation process may involve an alteration in the activity of plasma enzymes responsible for HDL remodeling, such as lecithin-cholesterol acyltransferase (LCAT) and/or cholesteryl ester transfer protein (CETP)[344,345]. In this regard, increased apoAI catabolism and the presence of smaller HDL particles in FH patients have been suggested to be the consequence of increased CETP activity along with reduced LCAT activity[346].

In addition, changes in HDL composition in terms of protein and lipid cargo can also be hypothesized[347]. For example, increased sphingomyelin (SM) and saturated fatty acid content, as well as increased cholesteryl ester and triglyceride content, were observed in HDL3 particles isolated from FH patients[348].

In the present study, we also observed an inverse association between HDL-CEC via the AD HDL-CEC process and ATX thickness, a result consistent with that observed by Ogura et al[245]. However, unlike ABCG1 HDL-CEC, AD-mediated HDL-CEC did not correlate with CLC, excluding a significant role of this efflux pathway in contributing to the higher CLC found in our FH patients with xanthoma.

In addition to promoting foam cell cholesterol accumulation, defective ABCG1 HDL-CEC may contribute to xanthoma formation by affecting inflammatory signaling in macrophages[153]. In this regard, a specific association between ABCG1 HDL-CEC dysfunction and inflammatory index has been reported in clinical studies[341,342,349]. In the context of tendon xanthoma formation, the observed decrease in ABCG1 efflux may increase the inflammatory status of macrophages within tendons, favoring foam cell formation. According to this hypothesis, macrophages from FH subjects with ATX showed a higher inflammatory status[350] and spontaneously released higher amounts of

inflammatory cytokines compared to cells from FH patients without ATX[351]. The impairment in ABCG1 HDL-CEC could partially be responsible of the higher CV risk that characterizes FH with xanthoma compared to other FH, as recently reported by the results from a study conducted in our laboratory in RA subjects in which ABCG1 HDL-CEC was inversely correlated with CV risk, independently of HDL-C levels and after correction for CV score and plaque number at baseline.

Summary Part I

In conclusion, we report for the first time an association between the presence of ATX in FH subjects and serum lipoprotein dysfunction, namely higher CLC. The increased CLC of sera from our FH-presenting ATX patients is not related to changes in plasma lipids, but rather to a reduction in ABCG1 HDL-CEC. These results provide evidence that lipoprotein dysfunction is involved in the physiopathology of ATX and, more generally, in the accumulation of cholesterol in peripheral tissues, although these observations need to be confirmed by more extensive studies.

Discussion Part II

From the results obtained in the AAA clinical setting, we observed a result that matched what was evaluated in the previous FH clinical setting, i.e. a significantly lower ABCG1 HDL-CEC and higher ABCA1 HDL-CEC in AAA patients compared to controls, both independent of HDL-C levels (the same in both groups of patients), suggesting a reduction or dysfunction of mature particles specific for ABCG1[343]. This is consistent with our hypothesis of accelerated HDL turnover. Indeed, the inverse correlation between ABCG1 HDL-CEC and ABCA1 HDL-CEC found only in the AAA group is consistent with the described mechanisms of HDL remodeling. In this regard, we also found an increased LCAT and CETP activity only in AAA patients, which may be related to the genetic background of AAA[352]. The direct correlation between the two enzyme activities detected only in the AAA group led us to hypothesize that in these patients, increased LCAT provides CETP with abundant substrate, i.e., HDL-esterified cholesterol, leading to accelerated HDL remodeling[324,353]. However, it cannot be excluded that such an association is also due to a genetic or epigenetic metabolic milieu affecting the expression of both enzymes in AAA patients[354]. Our data on LCAT and CETP activity differ from those presented in a previous report showing no difference between AAA patients and controls[267], possibly due to differences in the subject cohorts. The actual role of serum enzyme activity in this process is confirmed by the inverse and direct correlation of CETP activity with ABCG1 HDL-CEC and ABCA1 HDL-CEC, respectively. ABCG1-mediated efflux mainly occurs to mature HDL particles, which represent the buoyant plasma HDL, but it can also be promoted by discoidal pre-β migrating HDL with a size of ≥ 7.8 nm[355]. However, mature HDL are more efficient at accepting cholesterol from the ABCG1 transporter and are much more abundant in serum compared to pre-β HDL particles[324,343]. Thus, the ABCG1 HDL-CEC variations are likely related to the reduction and/or

dysfunction of mature HDL particles. On the other hand, we also observed lower serum pre-β particles in AAA compared to control patients, possibly due to increased HDL lipidation by LCAT.

The higher ABCA1 HDL-CEC that we found in AAA compared to control patients may be due to the activity of free apoAI as a cholesterol acceptor.

Although the final intracellular cholesterol content may be unaffected by the HDL-CEC changes we found in AAA patients, the reduction of ABCG1 HDL-CEC may be relevant for aneurysm development or expansion. Indeed, as already mentioned, cholesterol efflux through the ABCG1 transporter is coupled to intracellular anti-inflammatory signaling in macrophages and endothelial cells, and clinical studies have demonstrated a specific association between ABCG1 HDL-CEC impairment and inflammatory index[341,342,349]. Inflammation is considered to be a very important factor in vessel wall abnormalities during AAA formation[356]. The possible mechanisms linking the lipoprotein modifications we observed in AAA and aortic aneurysm pathology are discussed below.

Smoking is known to be a strong risk factor for the development of AAA[322]. Comparing HDL-CEC levels in smokers and nonsmokers, we found significant differences only in the AAA group. In smokers, ABCG1 HDL-CEC and ABCA1 HDL-CEC were significantly lower and higher, respectively, than in non-smokers. Although the very small number of patients included in this analysis does not allow conclusions to be drawn, these results suggest that smoking may accentuate HDL particle modifications specific to AAA patients. This concept is supported by a large clinical study in which smoking cessation was associated with an increase in serum mature HDL levels at 1 year[357]. However, the lack of effect of smoking on LCAT and CETP activity suggests the involvement of a different mechanism. The fact that smoking modulates HDL function exclusively in AAA patients suggests that genetic background may play an important permissive role in this group. The potential relevance of these findings is underlined by the fact that smoking and genetic background are the major risk factors for the development of AAA[358]. CLC did not differ between AAA and control patients, independently of LDL-C levels. On the one hand, the lack of difference in CLC between AAA and control patients is not surprising, since both groups were selected for the same cardiovascular risk/disease. However, this finding is relevant because it indicates that LDL alterations and dysfunction are not specific features of AAA patients.

Interestingly, only within the AAA group, serum CLC directly correlated with ABCG1 and AD HDL-CEC, mainly dependent on mature HDL, and inversely correlated with ABCA1 HDL-CEC and CETP activity. The positive correlation of CLC with mature HDL particles suggests that this HDL subpopulation may not only be dysfunctional with respect to HDL-CEC in AAA, but may even deliver cholesterol to cells[359]. The inverse correlation of CLC with ABCA1 HDL-CEC and CETP activity found only in AAA is again consistent with the existence of a link between accelerated HDL metabolism, intracellular cholesterol, and aneurysm formation.

Our data do not provide evidence that the alterations in HDL metabolism and function found in AAA patients have an impact on arterial tissue inflammation and repair. However, there is a large body of literature suggesting that altered HDLs induce changes in tissue homeostasis due to the failure of HDL-CEC. Such changes include the switching of macrophages to the proinflammatory and proapoptotic M1 phenotype[360,361], the control of angiogenesis[362], the regulation of smooth muscle cell function, and the secretion of metalloproteases[363]. In fact, inflammation is considered to be very important in the arterial wall changes leading to aneurysmal dilatation, and an altered polarization of arterial macrophages has been reported in AAA[364]. In addition, increased MMP-9 expression and elastic fiber destruction have been reported[356,365]. Dysfunctional HDLs are known to carry some inflammatory proteins that may activate processes involved in AAA development. One of these proteins, serum amyloid A (SAA), correlates with the abdominal aortic diameter in the early stages of AAA[366]. Indeed, SAA is involved in the regulation of leukocyte chemotaxis, inflammatory cytokine secretion, and MMP expression, thereby influencing extracellular matrix remodeling[367,368]. These processes are involved in AAA formation and progression. Consistently, a robust and inverse relationship has been documented between SAA HDL content and ABCG1 HDL-CEC, the specific pathway that was significantly decreased in our AAA patients[342]. Interestingly, in our results, as in a previous study[267], serum hs-CRP levels did not differ between AAA and control patients, suggesting that systemic inflammation does not play a major role in AAA formation. Our hypothesis is that in the context of a genetic background conditioning abnormal HDL remodeling and smoke sensitivity, with the contribution of factors such as smoke and local shear stress, chronic disturbances in abdominal aortic wall cholesterol fluxes and uncontrolled inflammation may contribute to arterial structural abnormalities and dilatation.

Summary Part II

In conclusion, these results demonstrate that AAA is associated with specific alterations in HDL metabolism and function, known to influence intracellular signaling of arterial wall cells, that are absent in vasculopathic patients with stenotic atherosclerosis. There is a great need to improve the early diagnosis and treatment of AAA before surgery is indicated. Although we could not demonstrate a causal relationship between lipoprotein alterations and aortic aneurysm development, our data suggest that future studies aimed at validating HDL-related parameters as diagnostic markers or therapeutic targets are worthwhile.

Discussion Part III

Regarding the neurodegenerative disease, in the presented cohort of AD patients, we have observed a reduced capacity of patients' HDLs to promote transporter-mediated cell cholesterol efflux through ABCA1 and ABCG1. These impairments could probably be due to an alteration of

cholesterol esterification that we evaluated, for the first time, in plasma of AD patients. Cholesterol esterification is a fundamental step in cholesterol transport in plasma and indeed 70% of circulating cholesterol is esterified. Additionally, these alterations strongly correlate with CSF AD biomarkers. The activity of LCAT may be another reason for the dysfunction reported in plasma HDL-CEC of AD. LCAT is the enzyme responsible for almost all circulating CEs, thus abnormalities in plasma cholesterol esterification parameters (i.e., unesterified cholesterol levels and unesterified/total cholesterol ratio) are likely to result from dysregulation of the LCAT system[369]. Indeed, we have found reduced LCAT activity in the plasma of AD patients, which could be explained by a selective reduction of plasma unesterified cholesterol, the LCAT substrate, in the presence of normal concentrations of total cholesterol. Unesterified cholesterol is carried by all plasma lipoproteins, but mainly by small discoidal HDL particles, which are selectively reduced in AD patients' plasma. Unesterified cholesterol in discoidal HDLs primarily derives from cellular cholesterol efflux via the active ABCA1- and ABCG1-mediated pathways[128].

Summary Part III

In summary, these results suggest that cholesterol esterification is impaired in plasma of AD patients and that plasma cholesterol esterification biomarkers (unesterified cholesterol and unesterified/total cholesterol ratio) are significantly associated with disease biomarkers (i.e., CSF $A\beta_{1-42}$). Such impairment is associated with defects in HDL functions, namely plasma cholesterol efflux capacity. Whether the dysregulation of cholesterol esterification is limited to AD or is a common trait in other neurodegenerative disorders remains to be defined. Taken together, the reported results suggest that LCAT-mediated cholesterol esterification may represent a potential target for novel therapeutic interventions in AD, although the causality between esterification abnormalities and associated cognitive decline cannot be established from our data. A final caveat should be made, since only about a third of plasma samples were obtained from cognitively intact controls who were characterized by normal CSF biomarkers. In fact, some control subjects, although asymptomatic, may express AD biomarkers many years before cognitive complaints may occur, which may partially bias our results.

Discussion Part IV

Completely consistent results were observed in FH in treatment with PCSK9-i. Specifically, we found that in FH patients, PCSK9-i therapy significantly improved ABCG1 and AD-mediated HDL-CEC, whereas it significantly reduced serum CLC. To our knowledge, this is the first study to evaluate the effect of PCSK9-i on the functional lipid profile, namely serum lipoprotein functions, in a cohort of FH patients. These effects could be of great clinical relevance in these subjects, since an impaired HDL-CEC[246] and an increased serum CLC have been previously documented[306]. Indeed, the

comparison between CEC and CLC levels of FH patients after PCSK9-i treatment and those of a small group of control sera included as internal standards in our experiments suggests that the treatment seems to be able to restore HDL-CEC and serum CLC to normal levels. Thus, the beneficial effect of PCSK9-i on the functional lipid profile may contribute to the restoration of normal lipoprotein functions and eventually to the CV benefit of these drugs beyond LDL-C reduction in subjects at high CV risk such as FH.

Regarding the blood lipid profile, the directional changes observed for total cholesterol and LDL-C after treatment with PCSK9-i were consistent with previous reports by Ge et al. who described similar results from PCSK9-i clinical trials in FH subjects[370]. These results were independent of smoking and hypertension. However, in contrast to what was reported by Ge and colleagues, in which PCSK9-i led to a slight increase in HDL-C and a reduction in triglycerides[370], in our cohort, treatment did not significantly change HDL-C and triglyceride levels, although the latter tended to decrease. This discrepancy may be due to the relatively small sample size in the current study or to specific intrinsic characteristics of our subjects.

In our study, the observed increase in ABCG1 HDL-CEC after PCSK9-i treatment occurred despite no change in HDL-C levels, suggesting that the increase in HDL-CEC may reflect a relative increase in the amount and/or efficiency of mature HDLs, which are the major cholesterol acceptors for this efflux pathway[343]. Thus, the significant improvement in HDL function by ABCG1 observed in this study may indicate that HDL remodeling towards mature particles[371] may occur after PCSk9-i treatment. Consistent with our findings, it has been previously reported that PCSK9-i treatment is associated with an increase in medium-sized HDL particles[372], which are responsible for HDL-CEC mainly through ABCG1[343].

Only two studies have investigated the effect of PCSK9-i on HDL function. Lappegård et al first investigated HDL-CEC on lipoprotein apheresis and subsequently on PCSK9 inhibition with Evolocumab in FH subjects, and found no changes in HDL-CEC during PCSK9-i treatment[373]. However, the sample size in this study was particularly small (three subjects), and the ex vivo HDL-CEC assessment was performed in a different cellular model and with a different methodological assay, suggesting the need for careful interpretation of their results.

More recently, Ying and colleagues analyzed the effects of Evolocumab and Atorvastatin on HDL-CEC in healthy normolipidemic subjects and found that Evolocumab decreased HDL-CEC of whole plasma, thus considering the mixture of all lipoproteins present in serum. As the authors suggested, this effect was likely related to the reduction in apoB-containing lipoproteins induced by the treatment. Looking at the effects of the drug on the ability of the isolated serum HDL fraction to promote ABCA1 and AD-mediated HDL-CEC, the authors did not observe a significant influence of the treatment on either efflux pathway[374]. In this regard, our data are consistent with the lack of a

significant effect of PCSK9-i treatment on ABCA1 HDL-CEC, however, different conclusions came from our study with respect to HDL-CEC by AD processes, which was improved along with ABCG1 HDL-CEC by PCSK9-i treatment. Compared to Ying's study, our results showed an increase in HDL-CEC after PCSK9-i treatment in a clearly different experimental setting, i.e., healthy normolipidemic subjects vs. FH subjects characterized by higher atherosclerotic burden and CV risk. This likely implies a different effect of treatment on the lipoprotein functional profile and a different impact of the therapeutic strategy in clinical practice.

In addition to its role in cholesterol homeostasis, ABCG1 HDL-CEC may also affect inflammatory signaling in macrophages[153]. This association is supported by the results of clinical studies that have demonstrated a specific association between ABCG1 HDL-CEC impairment and inflammatory index[341,342]. In light with these findings, the improvement in ABCG1 efflux induced by PCSK9-i treatment could ameliorate the inflammatory status that characterizes subjects with a high cholesterol burden, such as FH[375]. In line with this consideration, a recent study in a cohort of FH subjects found that PCSK9-i ameliorated systemic inflammation by promoting the activation of T-regulatory cells[376]. Thus, PCSK9 could also be considered as an intriguing new player in the immune-inflammation axis and its inhibition could be a primary target in a chronic inflammatory disease such as atherosclerotic processes, beyond the PCSK9-i related LDL-C reduction.

In this clinical setting, we observed for the first time a reduction in CLC in FH patients treated with PCSK9-i treatment, suggesting that the likely PCSK9-i related reduction in arterial wall foam cell formation may contribute to the proven CV benefit associated with this treatment[377].

Despite the reduction in LDL-C levels observed after PCSK9-i treatment, only a weak correlation between CLC and LDL-C levels was observed, suggesting a specific effect of treatment on the overall ability of serum to deliver cholesterol to cells (CLC). In fact, previous studies have documented that cholesterol accumulation is not always dependent on circulating LDL-C levels, but it may correlate with the quality and/or function of these lipoproteins[378]. Among LDL subclasses, oxidized LDL (oxLDL) has an enhanced ability to induce macrophage cholesterol accumulation[379]; of note, Evolocumab has previously been shown to significantly reduce circulating oxLDL and especially small LDL in patients with coronary artery disease[380,381]. In addition, PCSK9-i treatment reduced plasma levels of lipoprotein(a) (lp(a))[382,383], an atherogenic lipoprotein that is highly susceptible to oxidation and endothelial penetration, promoting foam cell formation[384]. Thus, it is likely that PCSK9-i could affect circulating apoB-rich lipoproteins not only quantitatively but also qualitatively, improving the overall lipid profile and possibly explaining the lower CLC observed in our cohort of FH subjects.

Summary Part IV

In conclusion, and fully consistent with the results just reported, PCSK9-i treatment significantly increased both ABCG1-mediated HDL-CEC and HDL-CEC by AD pathway and reduced serum CLC in a cohort of FH subjects. Our results clearly demonstrated that the beneficial effect of PCSK9-i treatment on lipoprotein functions may contribute to the CV benefit of these drugs beyond LDL-C reduction in subjects at high CV risk such as FH in clinical practice; however, a well-designed study with a larger cohort of subjects is needed to evaluate the effect of PCSK9-i therapy on these pathways in FH subjects.

Conclusions

In conclusion, the results observed in the three clinical settings, all potentially associated with high cardiovascular risk generally showed a dysregulation of lipoprotein function in terms of a reduced ability of HDL to mediate cholesterol efflux through the ABCG1 transporter, independently of HDL-C levels, which was recently found to be inversely correlated with CV risk in a cohort of RA patients, as mentioned above., and an increased ability of serum lipoproteins to promote cholesterol accumulation in macrophage cells, independently of LDL-C levels, suggesting an altered distribution of LDL subclasses in favor of the more atherogenic oxidized or small LDL subclasses. Accordingly, in FH after pharmacological intervention with PCSK9-i, an increase in ABCG1 HDL-CEC and a reduction in serum CLC, suggesting a beneficial effect of pharmacological treatment on lipoprotein function, which could contribute to the reduction in CV risk associated with this therapy. Overall, these results may contribute to the identification of novel functional parameters, to be used together with the traditional ones to better delineate the CV risk in these populations and to improve the knowledge about the effect of this pharmacological intervention on serum functional lipid profile. In addition, these parameters may represent in the future a tool to identify new pharmacological targets for the treatment of cardiovascular and neurodegenerative diseases, considering the recent results on CSL112, which increased cholesterol efflux but it didn't meet its primary efficacy endpoint of MACE reduction (ClinicalTrials.gov NCT03473223).

Declarations

Parts of this Ph.D. thesis have been included in four publications:

- “HDL Cholesterol Efflux and Serum Cholesterol Loading Capacity Alterations Associate to Macrophage Cholesterol Accumulation in FH Patients with Achilles Tendon Xanthoma”; Maria Pia Adorni, Marta Biolo, Francesca Zimetti, Marcella Palumbo, Nicoletta Ronda, Paolo Scarinzi, Paolo Simioni, Maria Giovanna Lupo, Nicola Ferri, Lorenzo Previato, Franco Bernini, Alberto Zambon; Int J Mol Sci. 2022 Jul 26;23(15):8255. doi: 10.3390/ijms23158255.

- “HDL metabolism and functions impacting on cell cholesterol homeostasis are specifically altered in patients with abdominal aortic aneurysm”; Maria Pia Adorni, Marcella Palumbo, Cinzia Marchi, Francesca Zimetti, Alice Ossoli, Marta Turri, Franco Bernini, Ivana Hollan, Jiří Moláček, Vladislav Treska, Nicoletta Ronda; Front Immunol. 2022 Sep 12:13:935241. doi: 10.3389/fimmu.2022.935241. eCollection 2022.

- “Plasma and cerebrospinal fluid cholesterol esterification is hampered in Alzheimer's disease”; Marta Turri, Elisa Conti, Chiara Pavanello, Francesco Gastoldi, Marcella Palumbo, Franco Bernini, Vittoria Aprea, Francesca Re, Alberto Barbiroli, Davide Emide, Daniela Galimberti, Lucio Tremolizzo, Francesca Zimetti, Laura Calabresi; AGAINST-AD Group; Alzheimers Res Ther. 2023 May 20;15(1):95. doi: 10.1186/s13195-023-01241-6.

- “Effects of PCSK9 inhibitors on HDL cholesterol efflux and serum cholesterol loading capacity in familial hypercholesterolemia subjects: a multi-lipid-center real-world evaluation”; Marcella Palumbo, Antonina Giammanco, Francesco Purrello, Chiara Pavanello, Giuliana Mombelli, Antonino Di Pino, Salvatore Piro, Angelo Baldassare Cefalù, Laura Calabresi, Maurizio Averna, Franco Bernini, Francesca Zimetti, Maria Pia Adorni, Roberto Scicali; Front Mol Biosci. 2022 Jul 19:9:925587. doi: 10.3389/fmolb.2022.925587. eCollection 2022.

The Ph.D. candidate contributed to *in vitro* experiment, statistical analyses and preparation of manuscripts Figures and Tables. The Ph.D. candidate contributed to the manuscripts writing and revision.

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