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The role of paraoxonase 1 in regulating HDL functionality during aging

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Abstract

Pharmacological interventions to increase HDL concentrations have led to disappointing results and have contributed to the emergence of the concept of HDL functionality. The anti-atherogenic activity of HDL can be explained by their functionality or quality. The capacity of HDL to maintain cellular cholesterol homeostasis and to transport cholesterol from peripheral cells to the liver for elimination is one of their principal anti-atherogenic activities. However, HDL possess several other attributes that contribute to their protective effect against cardiovascular diseases. HDL functionality is regulated by various proteins and lipids making up HDL particles. However several studies investigated the role of paraoxonase 1 (PON1) and suggest a significant role of this protein in the regulation of the functionality of HDL. Moreover, research on PON1 attracted much interest following several studies indicating that it is involved in cardiovascular protection. However, the mechanisms by which PON1 exerts these effects remain to be elucidated.

Keywords: HDL functionality, PON1, cholesterol efflux, aging
During the last 20 years, several studies have been interested in the link between paraoxonases (PON), and more particularly PON1, and the development of cardiovascular diseases (CVD). Results of animal studies have clearly demonstrated a beneficial role of PON1 by protecting against atherosclerotic plaque formation. In humans, epidemiological studies have provided convincing evidence for the involvement of PON1 in the protection against CVD. The decrease of PON1 paraoxonase activity is increasingly associated with a higher risk of incidence of cardiovascular complications. Moreover, the demonstration that diet interventions may enhance PON1 activity has generated a huge interest for the development of pharmacological agent to improve PON1 activity and particularly in the presence of cardiovascular risk factor. The beneficial effect of PON1 is explained in large part by its ability to regulate the atheroprotective properties of HDL. However, the mechanism of this regulation is not clearly established. Moreover, PON1 is only one among the numerous proteins forming HDL. In this review we will discuss the HDL concentration and HDL function in the protection against CVD. We will focus on the role of PON1 in the regulation of the functionality of HDL and the mechanisms by which PON1 may regulate the atheroprotective activities of HDL.

Biogenesis and HDL metabolism: concentration versus functionality

Pre-βHDL are considered the early forms of HDL particles. They result exclusively from the hepatic (~ 70%) and intestinal (~ 30%) secretion of apolipoprotein A-I (apoA-I) (Brunham et al. 2006; Timmins et al. 2005). ApoA-I interacts with the ABCAI transporter, which results in its lipidation and the generation of pre-βHDL, which in turn are gradually transformed into mature HDL by the action of a number of plasmatic enzymes (Chroni et al. 2003; Haghpassand et al. 2001). During their maturation, HDL are enriched in cholesterol that is esterified by lecithin cholesterol acyl transferase (LCAT) and transferred back to the liver for elimination (Tsompanidi et al. 2010). This normal HDL maturation process, which is called reverse cholesterol transport (RCT), is the only way that excess cholesterol in peripheral tissues can be transferred
to the liver to be metabolized. RCT is one of the main atheroprotective activities of HDL (Esterbauer et al. 1997; Glomset 1968; Johnson et al. 1991).

Epidemiological studies have shown that there is an inverse relationship between HDL concentrations and the risk of cardiovascular diseases (CVD) (Gordon et al. 1977). However, other studies raise doubts about the validity of this observation: (1) carriers of the apoA-I Milano mutation have low HDL concentrations and, paradoxically, enjoy a greater life expectancy and a total lack of atherosclerotic lesions (Alexander et al. 2009; Frikke-Schmidt et al. 2008; Roma et al. 1993); (2) the Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) study reported a high mortality risk with Torcetrapib™ despite a 4-fold elevation in normal HDL concentrations (Barter et al. 2007); (3) an analysis of data from the Framingham study revealed that 44% of CVD occur in subjects with normal HDL concentrations (Ansell et al. 2003); and (4) several in vitro studies have shown that there is a significant reduction in the anti-inflammatory and antioxidant activities of HDL during aging, even in the presence of normal HDL concentrations (Jaouad et al. 2006a; Khalil et al. 1998; Loued et al. 2013). The link between HDL concentrations and HDL functionality is thus in doubt (Sviridov et al. 2002), and HDL functionality is increasingly being seen as being more important than HDL concentrations with respect to cardiovascular protection (Khera et al. 2011).

HDL have a number of potential anti-atherogenic activities, although the relative importance of these activities remains a subject of debate. HDL possess antioxidant, anti-inflammatory, antithrombotic, and immunomodulatory activities and also regulate endothelial function (Figure 1). However the participation of HDL in RCT is one of the most important and least controversial anti-atherogenic activities of HDL. The RCT activity of HDL is determined in vitro by the measurement of cholesterol efflux capacity from macrophages and is inversely associated with both carotid intima-media thickness and the likelihood of angiographic coronary artery disease (Khera et al. 2011).
HDL cholesterol efflux capacity

Cholesterol efflux from macrophages and other tissues is the first and rate-limiting step of RCT (Wang X. et al. 2007). RCT is modulated by both the predisposition of cells to release cholesterol and by the ability of HDL to transport cholesterol to the liver for elimination (Yancey et al. 2003). Cholesterol efflux is mediated via three pathways. The first cellular efflux pathway is via the ATP-binding cassette transporters (ABCA1 and ABCG1). ABCA1 and ABCG1 are members of a large family of ATP-dependent transporters that share common structural motifs for the active transport of a variety of substrates. Lipid-poor apolipoproteins, particularly apoA-1, are the preferred cholesterol acceptors for ABCA1 (Wang N. et al. 2000). ABCG1 is another transporter that promotes mass cholesterol efflux from cells to mature HDL particles (HDL2 and HDL3) but not to lipid-poor apoA-I (Yvan-Charvet et al. 2010). ABCA1 and ABCG1 promote unidirectional cholesterol efflux to lipid-poor apoA-I and apoE, and HDL particles, respectively. The second cholesterol efflux pathway involves the scavenger receptor class B type I (SR-BI). The movement of free cholesterol via SR-BI is bidirectional and depends on the direction of the cholesterol gradient (Ji et al. 2011). The third cholesterol efflux pathway is aqueous diffusion. This process involves the desorption of free cholesterol molecules from donor lipid-water interfaces and the diffusion of these molecules through the intervening aqueous phase until they collide with and are absorbed by an acceptor (Berrougui et al. 2012). In addition to the interaction of apoA-1 and mature HDL particles with various membrane proteins, which mediates cholesterol exchange, other HDL-associated proteins also regulate the cholesterol efflux capacity of HDL, including lecithin-cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), and paraoxonase (PON1). These proteins also regulate the other anti-atherogenic activities of HDL. A reduction in their enzymatic activities or protein concentrations may thus significantly impact the functionality of HDL and their capacity to prevent the initiation of the atherosclerosis process.

Lecithin-cholesterol acyltransferase (LCAT)
LCAT is synthesized principally in the liver and circulates in the plasma associated with HDL (α-LCAT) and LDL (β-LCAT). LCAT esterifies free cholesterol with phosphatidylcholine, contributing to the formation of cholesteryl ester and lysophosphatidylcholine (Jonas 2000), the maturation of HDL, and the conversion of HDL from discoidal to spherical particles. LCAT thus plays an important role in maintaining serum cholesterol homeostasis by regulating cholesterol transport to the liver. Nevertheless, the role of LCAT in the pathogenesis of atherosclerosis remains a subject of debate. Animal studies have shown, on the one hand, that the over-expression of LCAT in transgenic mice significantly increases HDL concentrations and, surprisingly, the number and size of atherosclerotic lesions in these mice compared to wild-type mice (Mehlum et al. 1997). The increased atherosclerosis in mice over-expressing LCAT has been explained by the formation of dysfunctional HDL particles, which are less effective in mediating cholesterol transport to the liver for elimination (Berard et al. 1997). On the other hand, LCAT knockout mice are more protected against atherosclerosis than wild-type mice, even when fed a pro-atherogenic diet (Lambert et al. 2001). In human studies, The Copenhagen City Heart and The Copenhagen General Population studies, which included more than 50,000 subjects, showed that one of the four common variants of LCAT is responsible for low HDL concentrations (Haase et al. 2012). However, the significant reduction in HDL concentrations due to the LCAT variant has no effect on the incidence of CVD (Haase et al. 2012).

An α-LCAT mutation is at the origin of a rare metabolic disorder (fish-eye disease) associated with low HDL concentrations and a visual complication (Asztalos et al. 2007; Calabresi et al. 2005). Paradoxically, despite their very low HDL concentrations, individuals with this metabolic disorder have the same risk for CVD as normal subjects (Calabresi et al. 2009; Calabresi et al. 2012). Interestingly, even though the HDL concentrations of these individuals is very low, the ability of their HDL to mediate cholesterol efflux is not affected (Berard et al. 2001; Calabresi et al. 2009; Elkhalil et al. 1997; Tanigawa et al. 2009). In addition, LCAT-deficient plasma is as efficient as control plasma in mediating cholesterol efflux (Berard et al. 2001). These results may explain the absence of premature atherosclerosis in LCAT-deficient patients and indicate
that HDL functionality is more important than quantity in terms of cardiovascular protection.

**Cholesteryl ester transfer protein (CETP)**

CETP is a hydrophobic glycoprotein produced in the liver and secreted into the plasma where it is mainly associated with HDL (Tall 1993). The importance of CETP in HDL metabolism was first evoked based on a mutation in the Japanese population whose phenotype is associated with very high HDL concentrations and a lower incidence of coronary heart diseases (CHD) (Akita et al. 1994; Boekholdt et al. 2003; Inazu et al. 1990; Koizumi et al. 1985). This aroused much interest in developing a CETP inhibitor that would increase HDL concentrations. Torcetrapib was one of the first CETP inhibitors tested in the ILLUMINATE trial to determine whether it would decrease cardiovascular events in high-risk populations (Barter et al. 2007).

Torcetrapib, by inhibiting CETP, which normally transfers esterified cholesterol from HDL to other lipoproteins (LDL and VLDL), increased HDL concentrations by approximately 72% (Barter et al. 2007). However, despite significantly increasing HDL concentrations, Torcetrapib did not reduce cardiovascular risk, and the ILLUMINATE trial was terminated because of very serious side effects (Rader 2007).

Nevertheless, the results of the trial contributed to the emergence of the concept of the HDL functionality (or quality). There is increasing evidence that HDL concentrations do not reflect HDL functionality, which may be much more important than HDL concentrations in terms of cardiovascular protection (Hiura et al. 2009; Sviridov et al. 2008). Other studies showed that the 4-fold increase in HDL concentrations observed in Torcetrapib-treated patients does not improve HDL functionality in terms of the ability of HDL particles to meditate cholesterol efflux (Yvan-Charvet et al. 2007).

**Paraoxonase 1 (PON1)**

PON1 is a member of a multigene family that also includes PON2 and PON3. PON1 was initially studied for its capacity to neutralize organophosphate xenobiotics such as paraaxon, sarin, soman, VX, chlorpyrifos
oxon, diazoxon, and other related toxic compounds. Since all these compounds are non-physiological substrates, the physiological role of PON1 was not studied at the time. PON1 is a calcium-dependent serum esterase that is exclusively synthesized in the liver and is found in the bloodstream associated with HDL. Research on PON1 attracted much interest following several studies indicating that it is involved in cardiovascular protection (Shih et al. 1998; Tward et al. 2002). PON1-deficient mice are more susceptible to atherosclerosis, while the over-expression of PON1 in mice increases their resistance to CVD (Shih et al. 1998; Tward et al. 2002). PON1 also prevents the accumulation of oxidized lipids within LDL (Mackness M. I. et al. 1991). Several studies have contributed to understanding how PON1 prevents the atherosclerosis process and the associated clinical complications. The anti-atherosclerotic activities of HDL, especially their antioxidant and anti-inflammatory activities and cholesterol efflux capacity, have been attributed, in large part, to PON1. However, the mechanisms by which PON1 exerts these effects remain to be elucidated.

**Paraoxonase 1 and the antioxidant activity of HDL**

Antioxidant activity constitutes one of the anti-atherogenic properties of HDL. Several studies have shown that HDL prevent the oxidation of LDL both *in vitro* and *in vivo* (Bonnefont-Rousselot, Khalil, Gardes-Albert, et al. 1997). This antioxidant effect was attributed for a long time to vitamin E, particularly α-tocopherol, which is found in HDL. However, a comparison of the α-tocopherol content of HDL and other lipoproteins showed that HDL contain the lowest α-tocopherol content (less than one molecule of α-tocopherol per HDL particle compared to VLDL and LDL, which contain 12 and 45 molecules of α-tocopherol per particle, respectively) (Bonnefont-Rousselot, Khalil, Delattre, et al. 1997; Romanchik et al. 1995). This clearly indicates that α-tocopherol content alone cannot explain the antioxidant activity of HDL. A study by Shih et al. demonstrated that the antioxidant effect of HDL is due principally to PON1 (Shih et al. 1998). These authors showed that HDL from PON1 knockout mice lose their capacity to protect LDL against
oxidation, while enriching these HDL with PON1 restores their antioxidant activity (Shih et al. 1998). Moreover, mice lacking serum PON1 are more susceptible to organophosphate toxicity and atherosclerosis than wild-type mice (Shih et al. 1998). On the other hand, Tward et al. showed that HDL from mice over-expressing human PON1 are better able to protect LDL against oxidation and that these mice are more resistant to atherosclerosis (Tward et al. 2002). In addition, HDL from avian species, which lack paraoxonase activity, are unable to prevent LDL oxidation in the presence of copper ions (Mackness B. et al. 1998). We purified PON1 from the plasma of healthy subjects and determined the capacity of human PON1 to prevent the oxidation of LDL in vitro (Jaouad et al. 2006a). Interestingly, PON1 purified from human plasma and used at physiological concentrations (40 to 80 µg/mL) is more effective in protecting LDL against oxidation than 50 µM α-tocopherol, which is approximately twice its physiological concentration (Jaouad et al. 2006a). Nevertheless, the antioxidant activity of PON1 has been questioned in other studies, which attributed the activity to the contamination of PON1 with platelet-activating factor acetylhydrolase (PAF-AH) or with tergitol during its purification from plasma (Connelly et al. 2005; Teiber et al. 2004). However, further studies, including ours, showed that recombinant human PON1 protein also possesses strong antioxidant activity, which negates the hypothesis that the antioxidant activity is due to contamination by other plasma proteins (Brushia et al. 2001; Loued et al. 2012). We also showed that the oxidation of PON1 with oxygen free radicals produced by the gamma radiolysis of water or the incubation of PON1 with n-ethylmaleimide (NEM) significantly affects its antioxidant activity (Jaouad et al. 2006a). PON1 has a free sulfhydryl group at position Cys284 that may be involved in its antioxidant activity. The Cys284 residue in PON1 is one of the amino acids that is modified by oxygen free radicals and by the action of NEM (Jaouad et al. 2006a). Cys-284 is also the active site for the antioxidant activity of PON1 (Aviram et al. 1998). The oxidation of Cys-284 can also occur in vivo under oxidative stress conditions, which would affect the antioxidant activity of PON1 and the anti-atherogenic properties of HDL. In agreement with this, we showed that the antioxidant activity of PON1 is significantly reduced with aging and that this is due to a decrease in
the number of free sulfhydryl groups on the PON1 of elderly subjects compared to young subjects (Jaouad
et al. 2006b). The paraoxonase activity of PON1 is also lower in elderly subjects. However, this decrease
cannot be explained by a reduction in its plasma concentration or a decrease in HDL concentrations (Seres
et al. 2004). The oxidative stress conditions that characterize the aging process may explain the decrease
in PON1 paraoxonase activity in the elderly (Seres et al. 2004). The susceptibility of HDL to lipid
peroxidation also increases with age (Khalil et al. 1998). The fact that PON1 is exclusively associated with
HDL and that it exhibits antioxidant activity suggests that the increase with aging of the susceptibility of HDL
to lipid peroxidation is largely due to an alteration of PON1 activity (Seres et al. 1996). The decrease in
PON1 activity has an impact on HDL functionality and may explain, at least in part, the development of the
atherosclerosis process in the presence of cardiovascular risk factors such as aging, diabetes, obesity, and
renal failure (Bounafaa et al. 2014; Cherki et al. 2007; Gbandjaba et al. 2012).

**PON1 and inflammation**

PON1-deficient mice display increased vascular inflammation and thrombogenesis, suggesting that
PON1 has anti-inflammatory activity (Ng et al. 2008). PON1 also has phospholipase-like activity that allows
it to hydrolyze phosphatidylcholine core aldehydes and to produce lysophosphatidylcholine (LysoPC) and
free oxidized fatty acids (Ahmed et al. 2002; Loued et al. 2012). It has been suggested that the hydrolysis of
these oxidized phospholipids may explain the anti-inflammatory activity of PON1 (Ahmed et al. 2003).
Intriguingly, the hydrolysis of oxidized phospholipids in oxLDL contributes to the formation LysoPC and
oxidized free fatty acids, both of which are pro-inflammatory (Ahmed et al. 2002; Rozenberg et al. 2003;
Schilling et al. 2009). This raises the question of how PON1 exerts its anti-inflammatory activity. In a recent
study, we measured the capacity of PON1 to hydrolyze oxidized phospholipids in oxLDL and oxHDL (Loued
et al. 2012). The addition of PON1 to oxLDL contributed to the hydrolysis of oxidized phospholipids and
significantly stimulated the production of LysoPC (Loued et al. 2012). On the other hand, the addition of
PON1 to oxHDL resulted in a concentration-dependent reduction in the production of LysoPC (Loued et al. 2012). These results were confirmed by measurements of endothelial cell-associated intercellular adhesion molecule 1 (ICAM1) expression. The incubation of PON1 with oxLDL resulted in a significant increase in ICAM1 expression on endothelial cells, suggesting that PON1 possesses pro-inflammatory activity (Loued et al. 2012), while incubating PON1 with HDL resulted in a significant decrease in ICAM1 expression on endothelial cells, suggesting that PON1 also possesses anti-inflammatory activity (Loued et al. 2012). This discrepancy can be explained by the fact that the anti-inflammatory effect of PON1 depends on its association with HDL (Loued et al. 2012). PON1 interacts with other lipoprotein components to hydrolyze and inactivate oxidized phospholipids. These interactions appear to be an intrinsic property of HDL (Loued et al. 2012). α-LCAT may be one of the HDL-associated enzymes that interact with PON1 to reduce the pro-inflammatory effect of oxidized lipids. oxHDL alone has a pro-inflammatory effect as measured by ICAM1 expression on endothelial cells, while the enrichment of oxHDL with PON1 (20 µg/mL) reduces ICAM1 expression (Figure 2). Interestingly, the anti-inflammatory activity of PON1 increases when LCAT (5 µg/mL) is added to the mixture (Figure 2). We hypothesize that LCAT, due to its lysolecithin acyltransferase II (LATII) activity, may interact with PON1 by esterifying LysoPC with fragmented acyl groups from oxidized phospholipids, thus reducing the pro-inflammatory activity of HDL (Goyal et al. 1997; Subbaiah et al. 1996). PON1 also reduces monocyte chemotaxis and adhesion to endothelial cells (Ahmed et al. 2003), which may be due to the capacity of PON1 to inhibit the biological activities of oxidized phospholipids and their hydrolytic products (Ahmed et al. 2003). In addition to its capacity to hydrolyze oxidized lipids, PON1 also suppresses the pro-inflammatory response of macrophages. This activity may be mediated via its interaction with the SR-BI receptor on macrophages (Aharoni et al. 2013). We showed that the decrease in the enzymatic activity of PON1 in elderly subjects significantly affects its anti-inflammatory activity (Loued et al. 2013). HDL obtained from elderly subjects have a lower capacity to reduce ICAM1 expression on endothelial cells than HDL obtained from young subjects (Loued et al. 2013). The enrichment of HDL with
PON1 increases the anti-inflammatory activity of HDL as determined by the measurement of ICAM1 expression (Figure 2). Interestingly, extra virgin olive oil consumption enhances the enzymatic activity of PON1 and the anti-inflammatory activities of both PON1 and HDL (Loued et al. 2013).

**PON1 and the capacity of HDL to mediate cholesterol efflux**

Although the ability of HDL to mediate cellular cholesterol efflux and transport cholesterol to the liver for elimination is considered the main anti-atherogenic activity of HDL, very few studies have investigated the role that PON1 may play in this process. The results of one *in vitro* study suggested that PON1 stimulates cholesterol efflux from macrophages (Rosenblat et al. 2006) and that this efflux depends, in part, on the capacity of PON1 to increase LysoPC formation, which may stimulate HDL binding to macrophages (Rosenblat et al. 2006). PON1 purified from human plasma enhances cholesterol efflux from J774 macrophages and THP-1 macrophage-like cells (Berrougui et al. 2012). This effect increases with the level of expression of the ABCA1 transporter on macrophages, suggesting that PON1 may stimulate cholesterol efflux via an interaction with this transporter (Berrougui et al. 2012). ApoA-I is the only protein associated with HDL that is known to interact with the ABCA1 transporter and to initiate cholesterol efflux. De Beer et al. showed that, under inflammatory conditions, serum amyloid A (SAA) has an apoA-I-like effect by mediating cholesterol efflux via the ABCA1 and ABCG1 transporters (de Beer et al., 2011). Remaley et al. showed that ABCA1-mediated cellular binding to apolipoproteins and lipid efflux is not specific to apoA-I but can also occur with other apolipoproteins that contain multiple amphipathic helical domains (Remaley et al. 2001). In addition, the apoA-I mimetic peptide has no sequence homology with the apoA-I protein but mimics the class A amphipathic helixes and interacts with ABCA1 to promote cholesterol efflux via this pathway (Xie et al. 2011). PON1, like apoA-I and SAA, also contains an amphipathic α-helix with approximately 22 amino acids in its secondary structure that enables HDL particles to tightly bind to and stabilize PON1 and stimulate its activity (Gu et al. 2016; Harel et al. 2004; Perez-Jimenez et al. 2005). The
presence of this structure strengthens the hypothesis that an interaction between PON1 and ABCA1 mediates cholesterol efflux. We showed that PON1 purified from human plasma uses an apoA-I-like mechanism to modulate cholesterol efflux from rapid and slow efflux pools derived from the lipid raft and non-raft domains of the plasma membrane, respectively (Berrougui et al. 2012). PON1 also stimulates the expression of ABCA1 on macrophages (Berrougui et al. 2012), which may be due to the capacity of PON1 to hydrolyze oxidized phospholipids in oxLDL and to the formation of LysoPC (Ikhlef et al. 2016). A pre-treatment of oxLDL with PON1 followed by an incubation with cholesterol-loaded macrophages stimulates cholesterol efflux to HDL and apoA-I, inducing an over-expression of the ABCA1 transporter (Ikhlef et al. 2016). Ikhlef et al., who investigated the effects of PPAR gamma, LXR alpha, and ABCA1 inhibitors, showed that PON1 stimulates cholesterol efflux by over-expressing ABCA1 via the PPAR\(\gamma\)-LXR\(\alpha\)-ABCA1 pathway (Ikhlef et al. 2016). This process can occur in vivo. In fact, PON1 and oxLDL levels are significantly higher in atherosclerotic lesions (Mackness B. et al. 1997). The coexistence of PON1 and oxLDL decreases the atherogenicity of plaque by inhibiting the bioactivity of oxidized lipids (Cohen et al. 2014), while the up-regulation of ABCA1 expression on macrophages stimulates the efflux of cholesterol from these macrophages (Ikhlef et al. 2016). An investigation by Ikhlef et al. using transgenic mice that over-express human PON1 confirmed the involvement of this HDL-associated protein in the stimulation of RCT (Ikhlef et al. 2017). RCT is significantly higher in transgenic mice over-expressing PON1 than in wild-type mice (Ikhlef et al. 2017). In addition, HDL from PON1-Tg mice have a higher capacity to mediate cholesterol efflux than HDL from wild-type mice (Ikhlef et al. 2017). Moreover, macrophages from PON1-Tg mice also express significantly more ABCA1 transporter than macrophages from wild-type mice (Ikhlef et al. 2017). Thus, PON1 may stimulate cholesterol efflux via two mechanisms; the first is that PON1 interacts directly with ABCA1 transporter to allow the efflux of cholesterol to HDL particles (Berrougui et al. 2012) and the second mechanism suggests that PON1 induces ABCA1 overexpression thereby facilitating the efflux of cholesterol via this transporter (Berrougui et al. 2012). These in vitro and in vivo studies provided evidence of the
involvement of PON1 in the regulation of many of the anti-atherogenic activities of HDL and showed that PON1 also modulates HDL functionality.

PON1 and other HDL activities

PON1 has been also suggested to regulate the apoptotic activity of HDL. This process has been explained by the capacity of PON1, at basal condition, to enhance SR-BI receptor expression on macrophages via a mechanism implicating LysoPC formation. The increased atherosclerosis development observed in PON1-deficient mice was explained as a result of reduced SR-BI mediated HDL protection against apoptosis (Farid et al. 2012; Fuhrman et al. 2010). PON1 was also suggested to regulate the anti-apoptotic activity of HDL and this effect was explained by the capacity of PON1 to reduce HDL oxidation (Camps et al. 2010). Ferree et al. 2000 demonstrated that enhanced PON1 protein expression was associated with increased serum biomarker of antiapoptotic activity (Ferre et al. 2006). Some studies have also associated PON1 to the anti-thrombotic and anti-adhesion activities of HDL and advanced that this effect may be explained by the capacity of PON1 to protect against oxidation and protein modification. However, there is not enough convincing data to confirm the involvement of PON1 in the regulation of these activities of HDL (anti-apoptotic, anti-thrombotic, anti-adhesion activities).

Aging and HDL functionality

It is well established that LDL concentrations and the pro-atherogenic effects of LDL increase with aging (Abbott et al. 1983; Heiss et al. 1980; Khalil et al. 1996). Conversely, there is no clear agreement regarding the effect of aging on HDL concentrations (Frishman et al. 1992; Nikkila et al. 1990; Nikkila et al. 1991; Schaefer et al. 1989; Schaefer, Lamon-Fava, Johnson, et al. 1994; Wallace et al. 1992; Wilson et al. 1994).
While prospective studies have shown that plasma HDL concentrations decline with age, cross-sectional studies have indicated that mean HDL cholesterol concentrations remain the same, or even increase, with aging (Wallace et al. 1992). Several other studies have suggested that these variations are gender-dependent and that, in men, HDL concentrations remain unchanged with aging (Frishman et al. 1992; Schaefer, Lamon-Fava, Cohn, et al. 1994; Wallace et al. 1992) while they decrease in women starting at age 60 (Brown et al. 1993; Cheung et al. 2009; Kim et al. 2000). This decrease has been attributed to hormonal changes in postmenopausal women (Wilson et al. 1994). Changes in the body mass index, in addition to aging, may also be an important determinant of the decline in HDL concentrations in the elderly. Some studies have reported that centenarians have HDL concentrations similar to those of middle-aged healthy populations (Barbagallo et al. 1998). High HDL concentrations have even been reported as having a positive effect on longevity (Landi et al. 2007). However, other studies have reported lower HDL concentrations in centenarians (Baggio et al. 1998). This discrepancy has been attributed to differences in sampling procedures and the nutritional and functional status of the centenarians (Arai et al. 2004).

Pharmacological intervention studies aimed at increasing HDL concentrations as well as studies on genetic variants that influence HDL concentrations have raised considerable doubt about the relevance of the HDL concentration hypothesis (Ishigami et al. 1994; Ohta et al. 1995; Yamashita et al. 1990). There is now general agreement on the importance of HDL functionality (or quality) rather than HDL concentrations in preventing cardiovascular diseases (Khera et al. 2011; Stock 2011). However, little is known about how HDL functionality is affected during aging and what impact a decrease in functionality may have on the development of atherosclerosis in the elderly.

Oxidative modifications of LDL and HDL may play an important role in the pathogenesis of atherosclerosis. The oxidation of LDL contributes to the accumulation of cholesterol in macrophages, resulting in their transformation into foam cells. The oxidation of LDL also initiates an inflammatory response that exacerbates the atherosclerosis process (Ross 1999). We showed that the susceptibility of LDL to
oxidation increases significantly with aging (Khalil et al. 1996). The increase in the susceptibility to oxidation may be caused by biochemical changes that occur with aging, particularly a decrease in endogenous vitamin E in LDL and an increase in total polyunsaturated fatty acids (Khalil et al. 1996; Reaven et al. 1999). HDL from elderly subjects is more susceptible to oxidation and, more importantly, to a significant reduction in their capacity to prevent LDL oxidation (Jaouad et al. 2006a; Khalil et al. 1998). The reduction in the antioxidant activity of HDL has been attributed to a reduction in PON1 activity, which is approximately 43% lower in the elderly (Milochevitch et al. 2001; Seres et al. 2004). Another study in our laboratory showed that the anti-inflammatory activity of HDL also declines significantly with aging (Loued et al. 2013). Although we have not clearly proven the involvement of PON1 in the reduction the anti-inflammatory activity of HDL, some of our results support this hypothesis (Loued et al. 2013). We showed that enriching HDL with PON1 enhances the anti-inflammatory effect of HDL (Figure 2) and that stimulating PON1 paraoxonase activity improves the anti-inflammatory potential of HDL (Loued et al. 2013).

The ability of HDL to mediate cholesterol efflux is considered the main anti-atherogenic activity of HDL and is strongly and inversely associated with the severity of atherosclerosis, independently of HDL cholesterol levels (Khera et al. 2011). The capacity to mediate cholesterol efflux from macrophages is a metric of HDL functionality (Khera et al. 2011). We showed that HDL from elderly healthy subjects have a lower capacity to mediate cholesterol efflux (Berrougui et al. 2007). Our results suggested that the alteration of this HDL activity is due to oxidative modifications of apoA-1, which reduces its interaction with the ABCA1 transporter (Berrougui et al. 2007). However, the subsequent demonstration that PON1 is involved in the stimulation of the cholesterol efflux capacity of HDL suggests that the decrease in PON1 activity during aging may explain the alteration of the capacity of HDL from elderly subjects to mediate cholesterol efflux. Our results from an animal study showed that plasma obtained from 24-month-old mice (aged mice) has a significant lower capacity to mediate cholesterol efflux from J774 macrophages than plasma obtained from 4-month-old mice (young mice) (Figure 3). Interestingly, the overexpression of PON1 in mice significantly
increases the ability of plasma from both in young and aged mice to mediate cholesterol efflux and also reduces the age-related difference in the cholesterol efflux capacity of plasma (Figure 3). HDL$_3$ have a higher PON1 content than HDL$_2$ (Rozek et al. 2005). Interestingly, the decrease with aging in the capacity of HDL to mediate cholesterol efflux is more closely associated with HDL$_3$ than with HDL$_2$ (Berrougui et al. 2007). This provides additional support for the hypothesis that there is a relationship between PON1 activity and the capacity of HDL to mediate cholesterol efflux. The reduction in the capacity of HDL to mediate cholesterol efflux in acute coronary syndrome has also been associated with a reduction of PON1 activity (Bounafaa et al. 2014).

Our results thus clearly showed that there is a decrease in the main anti-atherogenic activities of HDL in the elderly, confirming that HDL functionality is altered with aging. The alteration of functionality is also involved in several risk factors for CVD, in particular in patients with chronic inflammation disorders, including diabetes mellitus (Morgantini et al. 2011) and atherosclerosis (Navab et al. 1997). Although several factors may be involved the alteration of HDL functionality, a number of lines of evidence point to the involvement of PON1 in the regulation of HDL functionality.

**HDL functionality and diet**

Dietary patterns have been shown to impair or improve HDL functionality. Nutrition studies on HDL functionality have focused on the antioxidant, anti-inflammatory, antithrombotic, vasodilatory, and RCT properties of HDL. We recently showed that consumption of extra virgin olive oil, one of the key components of the Mediterranean diet, improves the antioxidant and anti-inflammatory activities of HDL as well as the capacity of HDL to remove excess cholesterol from peripheral tissues. Interestingly, these improvements are accompanied by an increase in PON1 activity (Helal et al. 2013). Other studies have reported that diets rich in monounsaturated fatty acids (MUFA) are associated with an increase in PON1 activity (Ferretti et al. 2012). In terms of RCT, Treguier *et al.* reported that the efflux of cholesterol from macrophages to plasma...
as well as in vivo RCT are significantly impaired in hamsters fed a cholesterol-enriched diet (Treguier et al. 2011). Nishimoto et al. reported that C57B6/j mice fed fish oil displayed higher in vivo macrophage RCT and ex vivo efflux to apoA-I than C57B6/j mice fed soybean or coconut oils (Nishimoto et al. 2009). In addition to macronutrients, the effects of bioactive compounds on HDL functionality have been investigated. We showed in a recent study that plant-sourced phenolic compounds enhance the anti-atherogenic properties of HDL by reducing oxidative damage and improving the capacity to promote cholesterol efflux and the RCT process (Berrougui et al. 2015). Fruit and vegetable intake also modulates HDL functionality. The consumption of pomegranate juice increases serum PON1 activity and reduces HDL oxidation (Aviram et al. 2000). Other epidemiological studies have shown that vitamin E supplementation improves the RCT function of HDL in type 2 diabetic individuals (Asleh et al. 2008). On the other hand, the supplementation of the diets of healthy men with tomato or carrot juice has no effect on PON1 activity (Bub et al. 2005)

Conclusion

CVD is a major health problem, particularly among the elderly. The elderly account for almost 80% of deaths due to CVD (Garcia-Palmieri 2006; Lakatta 2002). One epidemiological study showed that 0.025 M step increases in HDL concentrations are associated with 2 to 3% step reductions in the incidence of CVD (Toth 2005). However, pharmacological intervention studies aimed at increasing plasma HDL concentrations have led to disappointing results with respect to cardiovascular protection. Given these results, HDL functionality is increasingly being seen as important, if not more so, than HDL concentrations. Understanding the parameters that regulate HDL functionality will make it possible to develop new strategies to reduce the pathogenesis of atherosclerosis, especially in the presence of a risk factor such as aging. There is some evidence to indicate that PON1 plays an important role in regulating HDL functionality.
However, further studies are required to determine the mechanisms by which PON1 improves HDL functionality and protects against CVD.

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Fig. 1: Summary of the anti-atherogenic activities of HDL

Fig. 2. Assessment of the anti-inflammatory effect of PON1 on Ea.hy926 endothelial cells and the interaction of PON1 with LCAT

Oxidized HDL (oxHDL) were generated by exposing HDL to oxygen free radicals produced by the gamma radiolysis of water. The oxHDL were pre-incubated with PON1 or with PON1 and LCAT for 4 h. They were then incubated with Ea.hy926 endothelial cells. PON1 was used at a concentration of 50 µg/mL and LCAT at a concentration of 5 µg/mL. Data are expressed as means ± SEM. Mean values were compared using one way ANOVA followed by Bonferroni’s multiple comparison post test. *** p < 0.001 with respect to oxHDL alone.

Fig. 3. PON1 overexpression in mice increases the capacity plasma to mediate cholesterol efflux from macrophages

J774 macrophages (1 x 10^6 cells/mL) were loaded with [3H]-cholesterol (2 µCi/mL) for 24 h. Plasma samples obtained from WT and PON1-Tg mice were treated with polyethylene glycol to precipitate ApoB-100-containing particles and were then incubated with J774 macrophages for 24 h. Cholesterol efflux (radiolabeled cholesterol released from cells) was calculated using the following formula: (radioactivity (cpm) in supernatant/radioactivity (cpm) in cells + medium) × 100. Data are expressed as means ± SEM. *p<0.03 and ***p<0.003.
Figure 2

![Bar chart showing ICAM expression (% vs base) for different conditions: Baseline, PON1, oxHDL, oxHDL + PON1, oxHDL + PON1 & LCAT.](chart.png)
Figure 3

Cholesterol efflux to J774 macrophages (% of radiolabeled cholesterol)

- Wild-type young mice
- Wild-type aged mice
- PON1-Tg young mice
- PON1-Tg aged mice

Significance:
- ***
- *

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