**Research Title**

**Investigating the mechanism and function of PON1 palmitoyl-protein thioesterase** **activity in the endothelial cell membrane**

**Keywords:**

Paraoxonase 1, PON1, HDL, Endothelium, Palmitoylation, Cholesterol efflux Atherosclerosis, CVD.

**Abstract**

The serum activity level of the high-density lipoprotein (HDL)-associated enzyme paraoxonase 1 (PON1) is inversely related to atherosclerosis and cardiovascular disease (CVD), understandably making it the focus of seven decades-long intensive research. However, the link between plaque formation and PON1 activity has never been fully understood. PON1 is expressed almost exclusively in the liver and is then transported by HDL to the peripheral tissues. The lipophilic nature of PON1 enables its easy exchange between the lipoprotein and cell membranes in a process dependent on the HDL receptor scavenger receptor class B, type 1 (SR-B1). In endothelial cells, PON1 binding to the cell membrane leads to its internalization by endocytosis and subsequent complete inactivation by lysosomal degradation. PON1 is a "promiscuous" (i.e., nonspecific) enzyme with an unusually broad list of possible substrates, but its actual function and substrate are still largely unknown. The enzyme requires a lipid environment and becomes completely inactive upon delipidation. However, when PON1 binds HDL, its active site faces the lipoprotein's core and is inaccessible to external substrates. Hence, the HDL-bound PON1 is inactive toward substrates outside the particle's lipid core and is rapidly degraded and becomes inactive upon internalization. Consequently, the enzyme is only active in the cell membrane during its transit from HDL to the cytoplasm. To assign a function to PON1, we have investigated whether it is a palmitoyl-protein thioesterase (PPT) that can hydrolyze the palmitoyl moieties of membrane proteins involved in HDL and cholesterol transport, such as SR-B1, ATP-binding cassette transporter A1 (ABCA1) or their neighboring caveola proteins to facilitate the release of HDL or trigger its endocytosis. We have been able to demonstrate that PON1 can hydrolyze palmitoyl-cysteine thioester bonds in vitro, has direct or indirect PPT activity in vivo, and can significantly decrease the presence of SR-B1 in the endothelial membrane. However, there are still unanswered questions regarding the mechanism and function of this PON1 putative PPT activity. Therefore, our specific aims in this grant proposal are: **1)** to elucidate the mechanism of PON1-induced PPT activity and identify possible substrates; **2)** to identify PON1's structural elements involved in its transition to the cell membrane and endothelial endocytosis; **3)** to measure the effect of PON1 PPT activity and endocytosis on the endothelial HDL-mediated cholesterol efflux; **4)** to measure the effect of PON1 PPT activity and endocytosis on the endothelial HDL transcytosis. Meeting these goals will help shed new light on the native function of PON1 and its involvement in atherosclerosis and CVD – hopefully contributing to a better understanding of this mortal ailment.

**A detailed description of the research program:**

1. **Scientific background**

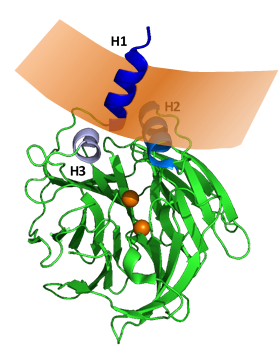
PON1 is a 354-amino acid, 43 kDa high-density lipoprotein (HDL)-bound enzyme belonging to a three-membered family of paraoxonases (reviewed by Furlong[1](#_ENREF_1)). Paraoxonases derive their name from the exclusive ability of PON1, discovered soon after WWII, to hydrolyze paraoxon, the toxic organophosphate ingredient of the insecticide parathion[2](#_ENREF_2). PON1 gained prominence only half a century later when Mackness and Walker first described its association with HDL[3](#_ENREF_3). Mackness *et al.* then went on to demonstrate, in 1991, that the enzyme was responsible for the ability of HDL to prevent the accumulation of oxidized lipids in low-density lipoprotein (LDL) and thus could be directly linked to atherosclerosis and cardiovascular disease (CVD) [4](#_ENREF_4). Since then, this direct link has been established by several epidemiological studies that have found an inverse relationship between PON1 serum activity and atherosclerosis and CVD (extensively reviewed in ref. [5-7](#_ENREF_5)).

PON1 mRNA is expressed almost exclusively in the liver and secreted into the circulation. Most PON1 (over 80%) circulates in the serum bound to HDL, but not equally to each subgroup. It does not bind nascent HDL at all and preferentially binds HDL3[8](#_ENREF_8),[9](#_ENREF_9). A small fraction of human serum PON1 is bound to VLDL [10](#_ENREF_10). Immunohistochemical analysis of mouse tissues has shown that the intracellular presence of PON1, unlike its expression, is unexpectedly widespread – probably due to its body-wide dissemination by HDL through the circulation [11](#_ENREF_11). However, it is still mostly unknown how PON1 makes its way from the liver to the peripheral tissues and how and when it binds HDL3. Deakin and James et al.[10](#_ENREF_10),[12](#_ENREF_12) have suggested that PON1 is extracted from the hepatocytes by a lipoprotein, which can then transfer it to HDL3[10](#_ENREF_10),[12](#_ENREF_12). They have shown that the process requires lipoprotein binding to the hepatic HDL receptor scavenger receptor class B type 1 (SR-B1) and probably a direct contact between the particle and the cell membranes[12](#_ENREF_12).

The atheroprotective capability of PON1 has been conclusively demonstrated by using PON1 knockout (PON1-KO, PON1-/-) and transgenic (PON1-Tg) mice. PON1-/- mice are more prone to developing atherosclerotic lesions and plaques than controls when fed a high-fat diet[13](#_ENREF_13). PON1 knockout alone cannot induce atherosclerosis in mice fed a balanced chow diet[13](#_ENREF_13). The development of atherosclerosis in PON1-/- mice requires either the high-fat diet or a second complete knockout of ApoE[14](#_ENREF_14),[15](#_ENREF_15). Conversely, mice overexpressing human PON1 are more resistant to atherosclerotic lesion development in a dose-dependent manner[16](#_ENREF_16). In both cases, however, the effect of PON1 expression level on serum HDL concentrations was insignificant or moderate[13](#_ENREF_13),[16](#_ENREF_16).

***PON1 structure:***

PON1 has a six-bladed beta-propeller architecture, with a catalytic site inside its central tunnel[17](#_ENREF_17). The catalytic site holds two calcium ions (Fig. 1), essential for its activity[17](#_ENREF_17). A depletion of the two ions completely inactivates the enzyme[18](#_ENREF_18). The access to the catalytic site is limited by a lid ("canopy") formed by two (out of three) alpha-helices (H2 and H3)[17](#_ENREF_17). The N-terminal alpha-helix (H1) is protruding above the catalytic site. It is probably used to anchor PON1 to the HDL particle by penetrating the lipid envelope of the latter like a tack[17](#_ENREF_17). The attachment of PON1 is assisted by a contact of the envelope with the surface of its second lipophilic helix. Together, both helices create a very high affinity (*K*d <<10-9) bond, with the catalytic site facing the lipoprotein[17](#_ENREF_17),[18](#_ENREF_18). This spatial position of the HDL-bound PON1 implies that it can hydrolyze only lipid-soluble substrates able to reach the active site by dissolving into the HDL's core[18](#_ENREF_18). Despite PON1's high affinity towards HDL, it is not a fixed component of the lipoprotein[19](#_ENREF_19). Its lipophilic nature allows it to be easily exchanged between VLDL and HDL and between HDL and cell membranes of culture cells (in vitro) or the arterial endothelium (in vivo)[19](#_ENREF_19).

The binding of PON1 to HDL, especially to ApoA1-HDL, dramatically increases its structural stability and catalytic efficiency towards lipophilic lactones [18](#_ENREF_18). The binding of PON1 to ApoE-HDL causes a similar increase in catalytic efficiency but has a lower capacity to stabilize the enzyme[20](#_ENREF_20). Delipidation of PON1 results in a complete loss of activity. Therefore, when HDL is not present in the reaction mixture in vitro, it must be replaced by detergents [18](#_ENREF_18). This absolute requirement of PON1 for a lipid environment is the reason why it is always associated with phospholipids - of either the plasma membrane or the lipoprotein's envelope [21](#_ENREF_21).

**Fig. 1: PON1 structure and interaction with HDL**

The three blue-shaded helices, H1, H2, and H3, sit above the catalytic site with its two calcium ions (orange spheres). The H1 and H2 hydrophobic regions of the protein are in contact with the HDL envelope (the light orange concave polygon). H1 protrudes into the lipid core of the lipoprotein[17](#_ENREF_17). This spatial arrangement blocks the catalytic site to external substrates.

***PON1 enzymatic activity:***

PON1 can hydrolyze, with varying efficiency, an unusually long list of potential substrates – a result of a decades-long quest to find its native substrate. The list includes numerous organophosphates, lactones, esters, and thioesters[22](#_ENREF_22). This unique quality of PON1 has been termed "promiscuity"[23](#_ENREF_23). It reflects the remarkable versatility of residues of the enzyme's catalytic group to perform multiple tasks depending on the substrate[22](#_ENREF_22). However, since it hydrolyses certain lactones with high efficiency, Khersonsky, and Tawfik believed that PON1 is a lactonase[22](#_ENREF_22). Mackness et al., who have discovered the ability of PON1 to protect LDL against lipid peroxidation by metal ions *in-vitro*[4](#_ENREF_4), have suggested that PON1 is a peroxidase and that its hydrolysis of LDL's lipid peroxides is the basis of the mechanism by which HDL protects against the development of atherosclerosis. This notion, supported by many publications claiming to corroborate it, has become entrenched in atherosclerosis research [24](#_ENREF_24).

Nonetheless, the exact mechanism by which PON1 protects against lipid peroxidation has not been adequately elucidated. It is clear whether it is the main catalytic site of PON1 that is involved in the antioxidant activity or whether LDL protection represents an independent second activity that relies on a free thiol group on cysteine 284. Aviram et al. (1998) have reported that inhibition of the main calcium-dependent catalytic site did not abolish PON1's ability to protect LDL against oxidation[25](#_ENREF_25). Conversely, blocking of the free cysteine 284 thiol caused a dose-dependent reduction in both the main catalytic activity of PON1 (i.e., paraoxonase and arylesterase) and the enzyme's inhibitory effect on LDL oxidation (i.e., peroxidase-like activity)[25](#_ENREF_25). Similar results have also been presented by Jaouad et al.[26](#_ENREF_26) and, later, by Tavori et al.[27](#_ENREF_27). However, since cysteine 284 is buried inside the protein's core, it is not clear how it affects both activities[17](#_ENREF_17).

At least two publications seem to refute the peroxidase activity of PON1 altogether. Connelly et al. and Teiber et al. have shown that purified PON1 was incapable of preventing LDL and HDL oxidation by either metal ions or peroxynitrite[21](#_ENREF_21),[28](#_ENREF_28). It could well be that this discrepancy only reflects some technical issues, such as impurities with antioxidant activity, which coeluted with PON1 during its purification in earlier experiments[21](#_ENREF_21). On the other hand, it may also point to some deeper problems with our understanding and cast doubt on the relevance of PON1's antioxidant activity to its genuine physiological function. In this regard, it is worth pointing out that attempts to find correlations between atherosclerosis and CVD and known PON1 polymorphisms, with varying catalytic activities and HDL binding affinities, yielded only poor results[7](#_ENREF_7). One notable example is the 192R/Q polymorphism. Gaidukov *et al.* have found that PON1 position 192 is involved in HDL binding and that the PON1-192Q variant binds HDL with a 3-fold lower affinity than the R isozyme.

Consequently, it exhibits significantly reduced stability, lactonase activity, and macrophage cholesterol efflux[29](#_ENREF_29). Nonetheless, no epidemiological study could find a significant correlation between these two variants and CVD. Only the general serum PON1 activity (measured as paraoxon or phenyl-acetate hydrolysis) can be correlated with an increased risk of developing atherosclerosis[7](#_ENREF_7).

***Other PON1 activities:***

Experiments that included intact macrophages or endothelial cells (ECs) have also provided hints that the function of PON1 is more complex and goes beyond mere hydrolysis of oxidized lipids. Several research groups have independently demonstrated that PON1 affects cholesterol biosynthesis, stimulates cholesterol efflux from macrophages and ECs (reviewed by Khalil et al.[6](#_ENREF_6)), and participates in SR-B1 signal transduction[30](#_ENREF_30).

Rozenberg et al. have shown, using PON1 deficient mice, that the enzyme inhibits cholesterol synthesis in mouse peritoneal macrophages (MPMs) and, to a lesser extent, in bovine ECs. The mechanism of this inhibition is not clear, although it is dependent on the integrity of PON1's cysteine 284[31](#_ENREF_31). Rosenblat et al. have found that PON1 also enhances the binding of HDL to the membrane of MPMs and the cholesterol efflux from these cells. They have attributed both effects to the increased production of lysophosphatidylcholine (LPC) by PON1 enzymatic activity[32](#_ENREF_32). The enhanced HDL binding and HDL-mediated cholesterol efflux were both found to be mediated by the ABCA1 transporter without the involvement of the HDL receptor SR-B1[32](#_ENREF_32). This result, however, is controversial as later studies have found that SR-B1 is essential for the cholesterol efflux from both mouse and human macrophages (reviewed by Linton et al.[33](#_ENREF_33)). The same research group has later demonstrated that PON1 specifically binds to the macrophage plasma membrane via binding sites, which are shared by HDL, and that the macrophage-bound PON1 is internalized and accumulates in the cytosolic compartment[34](#_ENREF_34).

Berrougui et al. have reported that enrichment of HDL3 with human PON1 enhanced, in a dose-dependent manner, cholesterol efflux from human THP1 macrophage-like cells and ABCA1-enriched mouse J774 macrophages. Interestingly, PON1 alone, without HDL3, could also mediate ABCA1-dependent cholesterol efflux from the macrophages. This ability may imply that PON1 shares structural and functional similarities with ApoA1[35](#_ENREF_35). In a series of additional extensive experiments, Berrougui et al. have also demonstrated that PON1 interacts with lipid rafts, directly binds ABCA1, can upregulate the expression of this lipid transporter (contrarily to Rosenblat et al.[32](#_ENREF_32)), and internalizes into the macrophages. Unfortunately, no mechanism has been proposed to suggest how PON1 achieves so many diverse feats[35](#_ENREF_35).

Ikhlef *et al.* have demonstrated that overexpression of human PON1 in mice substantially promotes the excretion of macrophage-derived cholesterol in the feces. They have also found that macrophages from PON1 overexpressing mice (PON1-Tg mice) liberate more cholesterol than macrophages from WT mice and express elevated levels of ABCA1[36](#_ENREF_36).

More recently, Gilad et al. have found that PON1 can penetrate human endothelial cells and significantly reduce the activity of calcium channels in the endothelium of murine mesenteric arteries[37](#_ENREF_37). Ben-David et al. have demonstrated that PON1 binds the membrane of cultured ECs via specific binding sites located in lipid-rafts/caveolae microdomains, which are shared with HDL and its receptor SR-B1[38](#_ENREF_38). This binding also leads to internalization, and therefore, this phenomenon is not unique to macrophages. In this case, the final intracellular destination of the internalized PON1 was the lysosome, and the process resulted in the complete loss of intracellular PON1 activity [38](#_ENREF_38).

***PON1 as a palmitoyl-protein thioesterase***

PON1 requires a lipid environment and becomes completely inactive upon delipidation [18](#_ENREF_18). However, when PON1 binds HDL, its active site faces the lipoprotein's core and is inaccessible to external substrates. Hence, the HDL-bound PON1 is inactive toward substrates outside the particle's lipid core and is rapidly degraded and becomes inactive upon internalization [38](#_ENREF_38). Consequently, the enzyme can be active towards external substrates only in the cell membrane during its transit from HDL to the cytoplasm. To assign a function to PON1, considering its unique characteristics, we have investigated whether it could be a palmitoyl-protein thioesterase (PPT) that can hydrolyze the palmitoyl moieties of membrane proteins involved in HDL and cholesterol transport and homeostasis, such as the HDL receptors SR-B1 and ABCA1, their neighboring caveola proteins or the HDL-activated endothelial nitric oxide synthase (eNOS[39-41](#_ENREF_39)). This hydrolysis could destabilize these membrane proteins, facilitating HDL release or triggering its endocytosis [42](#_ENREF_42).

So far, we have been able to show that PON1 can hydrolyze palmitoyl-cysteine thioester bonds in vitro, has direct or indirect PPT activity in vivo, and can significantly decrease the presence of the HDL receptor SR-B1 in the endothelial membrane [43](#_ENREF_43). We now believe that the exceptional promiscuity of PON1[23](#_ENREF_23) reflects a perfect adaptation to a large and virtually immobile substrate situated in the restrictive environment of the inner membrane leaflet. Under such conditions, the enzyme must maneuver around the substrate to correctly position the thioester bond with respect to the catalytic group. The flexibility of this catalytic group [23](#_ENREF_23) relaxes the restrictions on the correct relative spatial positions and increases PON1's ability to carry out the hydrolysis.

However, there are still many questions that need to be answered:

* Is PON1 a bonafide palmitoyl-protein thioesterase, or does it only activate an endogenous PPT[44](#_ENREF_44) when it traverses the cell membrane?
* Regarding the membrane proteins depalmitoylated by PON1 activity, is there a difference between the proteins depalmitoylated by the free PON1 and those depalmitoylated by the HDL-bound enzyme?
* How do PON1 structure and enzymatic activity affect its transit from the HDL particle to the endothelial cell membrane and its ability to penetrate it?
* Does PON1 affect the capacity of HDL (and also serum albumin) to efflux cholesterol from endothelial cells?
* Does PON1 affect HDL endocytosis and transcytosis?

1. **Research objectives and expected significance**

The proposed research aims to shed light on the function and mechanism of the newly discovered palmitoyl-protein thioesterase activity of PON1, on the function of this enzyme's unique ability to move from its carrier HDL and enter the endothelial cell, and on the link between these phenomena and the HDL-mediated endothelial cholesterol transport and efflux.

* 1. **The specific aims of the present study:**

1. **To determine whether PON1 can directly hydrolyze palmitoyl moieties of endothelial membrane proteins and identify proteins affected by PON1-induced PPT activity.** We will measure the hydrolysis of palmitoyl moieties by recombinant PON1 (rePON1) and its variants, with or without HDL, in vivo, in cultured wildtype arterial endothelial cells and matching cells incapable of expressing the endogenous PPT1. We will also use LC-MS-MS-based proteomics to identify the affected membrane proteins in each case. Achieving this aim will enable us to clarify whether PON1 directly hydrolyzes palmitoyl moieties and identify the targets of its PPT activity.
2. **To identify PON1's structural elements required for its endothelial endocytosis.** Using recombinant PON1 and its mutant variants, we will investigate the enzyme's structural elements required for its endocytosis and internalization. We will try to find out whether endocytosis is necessary for PON1 observed PPT activity or whether this activity is required for the enzyme's endocytosis.
3. **To investigate the effect of PON1 PPT activity on HDL-mediated cholesterol efflux in cultured arterial endothelial cells.** We will explore the effect of PON1 and its mutant variants on the cholesterol efflux rate and extent from arterial endothelial cells by various HDL subclasses (ApoA1, small HDL3, large HDL2, and whole HDL[45](#_ENREF_45),[46](#_ENREF_46)) at physiological concentrations in the presence or absence of the low-density lipoprotein LDL and human serum albumin (HSA)[47-49](#_ENREF_47), and the dependence of these two parameters on known lipoprotein receptors and cholesterol transporters. Achieving this aim will give us a better understanding of arterial endothelial efflux and the possible role played by PON1 and its PPT activity in this process.
4. **To investigate the possible effect of PON1 PPT activity on the intracellular transport of HDL by transcytosis in arterial endothelial cells.** We will assess the effect of PON1 and its mutant variants on the rate and extent of HDL endocytosis in arterial endothelial cells. Achieving this aim will enable us to identify a possible role of PON1 in intracellular HDL transport in arterial endothelial cells.
   1. **Expected significance:**

Achieving all four specified aims will provide enough evidence to validate our previous finding of PON1-induced PPT activity and support (or disprove) our hypothesis that PON1 native substrate(s) are palmitoyl residues of membrane proteins involved in HDL transport and cholesterol homeostasis and that the enzyme's promiscuity[23](#_ENREF_23) is merely a reflection of its unique adaptation to the restrictive environment of the inner leaflet of the cell membrane[43](#_ENREF_43). It will allow us to reliably assign, for the first time in decades, a substrate and function to PON1 and provide a coherent account of PON1 odd catalytic activity and distribution throughout the body, as well as its involvement in cholesterol homeostasis in vascular endothelial cells. These insights might provide new valuable information essential to our understanding of atherosclerotic plaque development.

1. **Detailed description of the proposed research**
   1. **The working hypothesis**

The working hypothesis of this proposal is that PON1 is a PPT enzyme with a unique adaptation for operation in, or in close vicinity to, the inner leaflet of the endothelial plasma membrane, where it affects the function of receptors, cholesterol transporters and other membrane proteins involved in HDL-mediated cholesterol transport and homeostasis, which are especially critical for the efficient reverse cholesterol transport (RCT) and clearance of excess intimal cholesterol and the prevention of atherosclerotic plaque formation[50](#_ENREF_50).

* 1. **Research design and methods**
     + 1. **Aim 1 – to determine whether PON1 can directly hydrolyze palmitoyl moieties of endothelial membrane proteins and identify proteins affected by PON1-induced PPT activity.**

We will determine whether PON1 is capable of directly hydrolyzing palmitoyl moieties of endothelial membrane proteins by four hours of incubation of arterial endothelial cells isolated from PPT1 KO[51](#_ENREF_51) and control wildtype mice (commercially available from The Jackson Laboratory, CA USA) with recombinant PON1 (rePON1 or its catalytically inactive double mutant variant rePON1-DM[52](#_ENREF_52), commercially available from The Weizmann Institute, IL)

The degree of palmitoyl residue hydrolysis will be assayed by the low-background fatty acyl biotinyl exchange (LB-ABE) method of Zhou et al.[53](#_ENREF_53), in which all free thiols are sequentially blocked, first by N-ethylmaleimide (NEM) and then by 2,2’-dithiodipyridine (DTDP). Palmitoyl residues (S-acylated sites) are then cleaved by hydroxylamine, leaving the freed cysteine thiols to react with a thiol-specific biotinyl-containing labeling reagent. The biotin labels then react with an avidin–horseradish peroxidase (HRP) conjugate. The number of thiol-specific labels is measured by quantifying the HRP reaction product using an enzyme-linked immunosorbent assay (ELISA) reader as previously described[43](#_ENREF_43).

The targets of PON1 thioesterase activity in the membranes of human aortic endothelial cells (HAEC) (from commercial primary culture or cell line) will be identified by combining the LB-ABE method with avidin-based affinity purification followed by extensive LC-MS/MS label-free proteomics, essentially as previously described by Mariscal et al.[54](#_ENREF_54). The proteomic analyses will be performed on affinity purified membrane proteins after incubating the cells with either free rePON1 or the HDL-bound enzyme. Since the presence of HDL is required to guide the enzyme to its SR-B1 receptor (while providing it a protective lipid envelope[43](#_ENREF_43)), a difference in the number and identity of proteins detected by this procedure is expected between the free enzyme and its HDL-bound counterpart.

**Expected results:**

The main expectation is that if PON1 directly hydrolyses palmitoyl residues, there will be no difference in depalmitoylation between PPT1 KO and wildtype murine arterial endothelial cells. We also expect that there will be no depalmitoylation after incubating the endothelial cells with the catalytically inactive double mutant rePON1-DM[52](#_ENREF_52)

The expectation from the palmitoyl-proteomics analyses is that there will be a difference in the identity and number of palmitoylated proteins detected after incubation of the endothelial cells with the free rePON1 compared to incubation with HDL-bound enzyme. We believe that the free enzyme can indiscriminately hydrolyze palmitoyl residues while the HDL-bound enzyme will be targeted to the HDL receptor SR-B1 and its associated proteins, resulting in a smaller number of palmitoylated proteins identified.

**Possible pitfalls:** A possible pitfall is that PON1 only triggers the hydrolysis of palmitoyl residues by activating PPT1[55](#_ENREF_55), which is also controlled by reversible cysteine acylation[56](#_ENREF_56). In this case, we will repeat the experiments, but this time using a specific human PPT1 inhibitor GNS561 (ezurpimtrostat)[57](#_ENREF_57) to see if PPT1 is the real PON1 target.

* + - 1. **Aim 2 – to identify PON1's structural elements required for its endothelial endocytosis.**

We will use HDL3-bound, fluorescently labeled rePON1, rePON1-DM, and PON1 lacking most of its N-terminal alpha-helix (H1) (PON1-Δ20, The Weizmann Institute, IL[17](#_ENREF_17)). fluorescence confocal microscopy, endocytosis inhibitors, and a lysosome-specific fluorescent dye to track rePON1 during its internalization into cultured human arterial endothelial cells as previously described[38](#_ENREF_38). This tracking will allow us to determine whether PON1 PPT activity is required for its internalization by endocytosis. We will use unlabeled HDL3-bound rePON1 variants and endocytosis inhibitors to determine whether the observed PON1-dependent depalmitoylation requires and coincides with PON1 endocytosis. If PON1-Δ20 is incapable of internalization, we will use site-directed mutagenesis to determine which residues in PON1's N-terminal first 20 positions are essential for its internalization.

**Expected results:** PON1 PPT activity is independent of its endocytosis but not vice versa.

**Possible pitfalls:** The bulky fluorescent label interferes with PON1 PPT activity. In this case, we will label PON1 in situ using fluorescently labeled anti-PON1 antibodies after fixation and permeabilization of the cells[38](#_ENREF_38).

* + - 1. **Aim 3 – to investigate the effect of PON1 PPT activity on the HDL-mediated cholesterol efflux in cultured arterial endothelial cells.**

We have already shown that PON1 can reduce the total HDL-mediated cholesterol efflux from human umbilical vein endothelial cells (HUVECs). This efflux is partially dependent on the HDL receptor SR-B1[43](#_ENREF_43). However, we now wish to investigate the effect of PON1 on the HDL-mediated efflux from the more relevant human arterial endothelial cells (HAECs) and measure its total capacity and rate. These two endothelial HDL-mediated efflux parameters will be determined by first loading confluent HAECs with fluorescently labeled cholesterol (using a commercial kit and protocol) and then measuring, at 30 min intervals for 4 h, the release of labeled cholesterol into the media as it is induced by various HDL subclasses (ApoA1, small HDL3, large HDL2, and whole HDL[45](#_ENREF_45),[46](#_ENREF_46)), at physiological concentrations, with or without PON1 or its mutant variants (rePON-Δ20[17](#_ENREF_17) and rePON1-DM[52](#_ENREF_52)). Since LDL and human serum albumin (HSA) are involved in endothelial efflux[47-49](#_ENREF_47) and LDL also competes with HDL for SR-B1 binding[58](#_ENREF_58), we will also investigate their effect on the endothelial HDL-mediated efflux and PON1 activity. The dependence of possible PON1 effects on SR-B1 will be explored by manipulating the receptor's expression by transient transfection of its mRNA or specific commercial siRNAs. Achieving this aim will give us a better understanding of arterial endothelial efflux and the possible role played by PON1 and its PPT activity in this process.

**Expected results:** Faster HDL3-mediated arterial endothelial efflux in the presence of PON1.

**Possible pitfalls:** The manipulation of SR-B1 expression by siRNA might be difficult. In this case, we will use anti-SR-B1 antibodies as previously described [43](#_ENREF_43).

* + - 1. **Aim 4 – To investigate the possible effect of PON1 PPT activity on the intracellular transport of HDL by transcytosis in arterial endothelial cells.**

The endothelial transcytosis of HDL is construed as an RCT mechanism for increased cholesterol clearance from the intima. HDL transported by transcytosis can efflux excess cholesterol from intimal macrophages and return it to circulation by transcytosis in the basolateral-to-apical direction[59](#_ENREF_59),[60](#_ENREF_60). Vaisman et al.[59](#_ENREF_59) speculated that cholesterol from intimal HDL particles can be taken up by basolateral SR-B1 receptors and then transported across the endothelial cells as free cholesterol, to be picked up again by apical SR-B1 receptors and plasma HDL. Therefore, we will measure the bidirectional (i.e., apical-to-basolateral and basolateral-to-apical) transcytosis HDL and the effect exerted by PON1 or its mutant variants on this process. The measurement will be carried out, essentially as previously described[61](#_ENREF_61), by using a confluent monolayer of HAECs in a Transwell assay system and fluorescently labeled lipoproteins, with or without PON1.

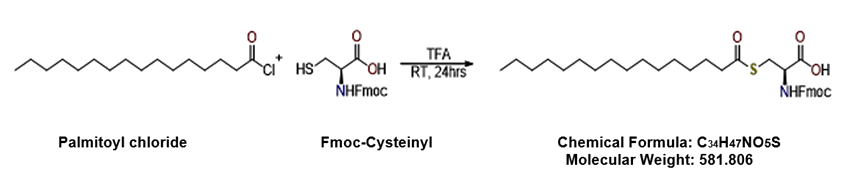
Since LDL competes with HDL for SR-B1[62](#_ENREF_62), which is a major component of the transcytosis apparatus, physiological concentrations of unlabeled LDL will be present in both transwell chambers to make sure that the observed HDL transcytosis and PON1 effects are not an artifact of low LDL concentrations. The presence of labeled lipoproteins in the opposite (upper or lower) transwell chamber will be measured by a spectrofluorometer.

**Expected results:** An observable effect of PON1 PPT activity on HDL transcytosis.

**Possible pitfalls:** transwell experiments are problematic because manipulating endothelial monolayers often introduces paracellular or intercellular gaps, confounding the analysis of transcytosis[61](#_ENREF_61),[62](#_ENREF_62). To avoid this pitfall, we will use the method of Bian et al.[61](#_ENREF_61), where an identical control transwell culture, with a six-fold excess of unlabeled lipoprotein, is added to each experiment. The unlabeled lipoprotein can mask any receptor-mediated transcytosis of labeled lipoproteins and allows the measurement of only paracellular passage. To reduce the lipoprotein-free label background in the bidirectional transcytosis experiments, the samples will be dialyzed before measurement.

* 1. **Preliminary results**
     1. **Substrate synthesis and PON1 PPT activity in vitro**

The palmitoyl thioesterase activity of rePON1 was measured in vitro using the synthesized substrate, cysteinyl palmitate thioester, in which the cysteine's amine is protected by the Fmoc (fluorenylmethoxycarbonyl) protection group. The substrate was synthesized, purified, and identified using LC-MS/MS and 1H-NMR (Fig. 2).



**Fig. 2: The synthesis process of rePON1 PPT activity substrate**

The ability of rePON1, with and without HDL, to cleave the thioester bond of the substrate was measured spectrophotometrically using Ellman's assay. Incubating the substrate with either rePON1 or HDL alone did not result in substrate cleavage. However, the addition of rePON1 after overnight incubation of HDL with the substrate to allow its diffusion into the lipoprotein resulted in a statistically significant increase in substrate degradation, probably due to increased stability and activity of PON1 in the phospholipid envelope of HDLs[18](#_ENREF_18) (Fig. 3).



**Figure 3: rePON1 hydrolysis of a synthetic substrate**

Palmitoyl-protein thioesterase (PPT) activity of rePON1 (100 µg/ml) with palmitoyl-Fmoc-cysteinyl thioester at 1 mM concentration. rePON1 was added separately or after incubating the substrate with HDL overnight at 37°C. PPT activity was measured using Ellman's reagent and calculated as µM TNB produced per minute; the extinction coefficient of TNB at 412 nm is 14150 M-1cm-1. Results are presented as mean ± standard error (n = 3). \*\*\*\* Significant difference between substrate incubated with rePON1 and HDL versus substrate incubated with rePON1 or HDL separately, *p* < 0.00001.

* + 1. **PON1 PPT activity in vivo**

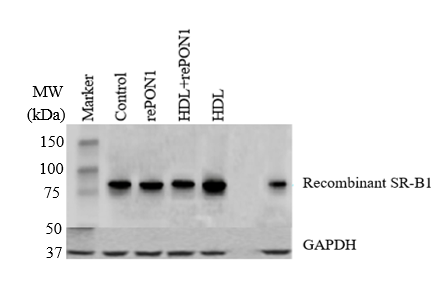
Human umbilical vein endothelial cells (HUVEC) were incubated with rePON1 (10 µg/ml), HDL (50 µg/ml), or both for 4 h. The cells were then washed, and protein lysates with the same adjusted protein concentration were prepared. The number of protein thioester bonds was measured spectrophotometrically using the acyl biotin exchange (ABE) ELISA method[63](#_ENREF_63). Incubating the cells with rePON1 alone significantly reduced the number of protein thioester bonds. The addition of the PON1 inhibitor 2HQ with rePON1 abolished this activity. Incubating the cells with HDL alone significantly increased the number of protein thioester bonds. In contrast, adding rePON1 with HDL significantly decreased the number of protein thioester bonds compared to incubation with HDL alone (Fig. 4). Here, again, the addition of the PON1 inhibitor abolished this effect. The results suggest that rePON1 triggers the breakdown of protein thioester bonds in vivo or somehow inhibits their increase by HDL.

**Fig. 4: rePON1 decreases the quantity of palmitoylated cysteine residues in HUVEC.**

HUVEC were incubated with PON1, HDL, or both for 4 h and then collected and lysed. All cell lysates had the same adjusted protein concentration. The number of thioester bonds in the cell lysates was measured spectrophotometrically at 650 nm by ABE–ELISA assay. Results are presented as mean ± standard error (n = 3). \*, \*\*, \*\*\*, \*\*\*\* Significant difference at *p* < 0.05, 0.01, 0.001, and 0.0001, respectively.

* + 1. **PON1 effect on endothelial SR-B1 expression**

The effect of rePON1, HDL, or both on SR-B1 expression in HUVEC endothelial cells was measured by western blot using either anti-SR-B1 or anti-GAPDH (as the normalizing protein) primary antibodies and goat anti-rabbit HRP secondary antibodies. Incubating the cells with HDL significantly increased SR-B1 expression compared to the control. However, the addition of rePON1 together with HDL significantly decreased SR-B1 expression compared to cells treated with HDL alone (Fig. 5A and B).



**Fig. 5: Western blot analysis of rePON1's effect on SR-B1 expression in HUVEC lysate**

**A**

**B**

Cell lysates were prepared from HUVEC treated with rePON1 (10 µg/ml), HDL (50 µg/ml), or both. The cell lysate proteins (10 g) were separated by 10% SDS-PAGE and analyzed by ECL-based Western blot. (**A**) SR-B1 and GAPDH (control) expression. (**B**) Quantification of SR-B1 expression normalized to GAPDH. \*\*, \*\*\* Significant difference at *p* < 0.01 and 0.001, respectively.

* + 1. **PON1 effect on SR-B1 membrane presence**

The effect of rePON1, HDL, or both on SR-B1 presence on endothelial cell membranes was measured by direct ELISA. HUVEC cells, grown on an ELISA plate, were incubated with rePON1, HDL, or both and reacted with anti-SR-B1 rabbit antibodies after washing and fixation. Specific binding was measured calorimetrically at 412 nm. As before, incubation with HDL significantly increased SR-B1 presence on the cell membrane compared to control cells without treatment. The addition of rePON1 together with HDL significantly decreased SR-B1 membrane presence (Fig. 6).

**Fig. 6: Effects of HDL and rePON1 on the presence of SR-B1 on cell membranes**

The effect of rePON1, HDL, or both on the presence of SR-B1 on endothelial cell membranes was measured by direct ELISA. Endothelial cells were incubated with rePON1 (10 µg/ml), HDL (50 µg/ml), or both for 6 h. The cells were then fixed, blocked, and reacted with primary anti-SR-B1 and secondary goat anti-rabbit HRP antibodies. Results are presented as mean ± standard error (n = 3). \*, \*\*\*, \*\*\*\* Significant difference at *p* < 0.05, 0.001 and 0.0001, respectively.

* 1. **Facilities available to the researchers**

The proposed research will be carried out at the Laboratory of Natural Compounds and Analytical Chemistry at MIGAL Galilee Research Institute under the supervision of Prof. Soliman Khatib. The laboratory specializes in medicinal chemistry, the characterization of proteins, and small molecule interactions using experimental and molecular modeling methods, in addition to synthesizing, isolating, identifying, and quantifying bioactive natural compounds with biological activities, especially compounds that increase PON1 activity and improve HDL quality. The laboratory also specializes in in vitro assays related to atherosclerosis using endothelial and macrophage cells. The laboratory is fully equipped with all the facilities and instrumentation required for the proposed experiments: Infinite M200 multimode microplate readers, centrifuges, chemical and biological hoods, cell culture rooms, cell incubators, cell culture equipment, refrigerators, freezers, computers, FACS, and confocal and fluorescence microscopes. Analytical instruments include HPLC, HR-LCMS, and GC-MS-MS. The laboratory personnel includes four Ph.D. students, three M.Sc. students, and a technician.

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