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Short Communication

Relevance of the amino acid conversions L144R (Zaragoza) and L159P (Zavalla) in the apolipoprotein A-I binding site for haptoglobin

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Abstract

The high-density lipoprotein apolipoprotein A-I (ApoA-I) stimulates the enzyme lecithin-cholesterol acyltransferase (LCAT) in the reverse cholesterol transport pathway. Two ApoA-I variants, Zaragoza (L144R) and Zavalla (L159P), are associated with low levels of HDL-cholesterol but normal LCAT activity. Haptoglobin interacts with ApoA-I, impairing LCAT stimulation. Synthetic peptides matching the haptoglobin-binding site of native or variant ApoA-I (native, P2a; variants, Zav-pep and Zar-pep) bound haptoglobin with different activity: Zar-pep>P2a>Zav-pep. They also differently rescued LCAT *in vitro* activity in the presence of haptoglobin (P2a=Zar-pep>Zav-pep). Therefore, both amino acid conversions affect haptoglobin binding and LCAT regulation. We highlight the role of haptoglobin in LCAT regulation in subjects with ApoA-I variants.

Keywords: ApoA-I Zaragoza; ApoA-I Zavalla; high-density lipoprotein; lecithin-cholesterol acyltransferase; peptides; reverse cholesterol transport.

Apolipoprotein A-I (ApoA-I), the major apolipoprotein of high-density lipoprotein (HDL), plays a key role in the removal of excess cholesterol from peripheral cells in a recognized anti-atherosclerotic process called reverse cholesterol transport (RCT) (Fielding and Fielding, 1995). In particular, ApoA-I stimulates the enzyme lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43) to convert excess cholesterol released from peripheral cells into cholesteryl esters that are embedded in HDL for transport in the circulation (Fielding and Fielding, 1995). Therefore, ApoA-I-dependent regulation of LCAT is a crucial step in the transport of cellular excess cholesterol to the liver for elimination.

Several naturally occurring ApoA-I mutations are associated with pathological phenotypes (Miettinen et al., 1997; Huang et al., 1998; Miller et al., 1998; Sorci-Thomas and Thomas, 2002; Hovingh et al., 2004; Zannis et al., 2006). They may affect different steps in the biogenesis of HDL, cause dyslipidemia, or influence plasma HDL levels (Yamakawa-Kobayashi et al., 1999; Zannis et al., 2008). A total of 46 ApoA-I variants have been reported in humans to date: 25 mutations are associated with low HDL levels and 17 mutations cause reduced capacity of ApoA-I to activate LCAT (Sorci-Thomas and Thomas, 2002; Zannis et al., 2006). In particular, mutations within helices 5, 6, and 7 in the apolipoprotein structure are strongly associated with lower LCAT activity in plasma. It has been suggested that this phenomenon results from conformational changes that alter the ability of ApoA-I to stimulate the enzyme (Sorci-Thomas and Thomas, 2002). These mutations span the region from residue 107 to residue 235 of the ApoA-I structure, and most of them are clustered predominantly on or in the vicinity of helix 6 (Zannis et al., 2006). Interestingly, it has been shown that some of these mutations predispose subjects to atherosclerosis (Miccoli et al., 1996; Miettinen et al., 1997; Miller et al., 1998; Huang et al., 1998; Hovingh et al., 2004). Among ApoA-I variants, two with a single amino-acid change (namely ApoA-I Zavalla and ApoA-I Zaragoza, herein denoted Zav-ApoA-I and Zar-ApoA-I, respectively) are associated with low levels of cholesteryl esters in plasma HDL (Miller et al., 1998; Recalde et al., 2001). Despite reduced cholesterol esterification and transport, subjects with Zar-ApoA-I had no signs of cardiovascular disease (CAD) (Recalde et al., 2001). Similarly, symptomatic CAD was reported for only two subjects with Zav-ApoA-I, but they displayed additional coronary risk factors, including cigarette smoking and hypercholesterolemia (Miller et al., 1998). In both Zar-ApoA-I and Zav-ApoA-I subjects, the plasma cholesterol esterification rate and LCAT activity, as assayed *in vitro*, were not significantly different from those in control subjects (Miller et al., 1998; Recalde et al., 2001). This finding suggests that the ApoA-I-dependent regulation of LCAT activity *in vivo* might be influenced by the amino acid variations L159P and L144R in Zav-ApoA-I and Zar-

ApoA-I, respectively. Both mutations are in the ApoA-I domain that is indispensable for LCAT stimulation, a region that includes helix 6 and spans residues 143–186 in the protein sequence (Sorci-Thomas et al., 1998; Sorci-Thomas and Thomas, 2002). It is noteworthy that some ApoA-I mutations in this domain, namely ApoA-I Oita (V156E), ApoA-I Pisa (L141R), ApoA-I Fin (L159R) and ApoA-I Seattle (lacking residues 146–160), are associated with poor cholesterol esterification by LCAT (Sorci-Thomas and Thomas, 2002). It has also been demonstrated that negatively charged amino acids within ApoA-I helix 6 directly modulate the catalytic efficiency of LCAT (Alexander et al., 2005). This ApoA-I domain required for LCAT stimulation can be bound by haptoglobin (Hpt), an acute phase glycoprotein that captures and transports free hemoglobin (Hb) to the liver (Langlois and Delanghe, 1996). The high Hpt levels present during inflammation impair LCAT activity and it has been suggested that this inhibition depends on competition between Hpt and LCAT for ApoA-I binding (Balestrieri et al., 2001; Cigliano et al., 2001; Spagnuolo et al., 2003, 2005). Such a role for ApoA-I-Hpt binding, although interfering with ApoA-I-mediated stimulation of LCAT, might be important in protecting the apolipoprotein functional region from oxidative damage (Salvatore et al., 2007). *In vitro* experiments demonstrated that an ApoA-I-derived peptide (P2a, ApoA-I sequence from L141 to A164) can displace Hpt from ApoA-I and therefore rescue ApoA-I function in cholesterol esterification (Spagnuolo et al., 2005). This information prompted the hypothesis that low cholesterol esterification in subjects with Zav-ApoA-I or Zar-ApoA-I might depend on higher than normal inhibition of LCAT by Hpt. To test this hypothesis, peptides with the ApoA-I sequence L141–A164 harboring L159P (Zav-pep) or L144R (Zar-pep) were synthesized and used to mimic the function of the ApoA-I variants *in vitro*. The aim of this work was to investigate whether these peptides interact with Hpt or compete with native ApoA-I or its mimetic peptide P2a in Hpt binding. The ability of these peptides to rescue LCAT activity *in vitro* in the presence of Hpt was expected to be in agreement with their ability to bind Hpt.

To investigate whether the gene mutations expressing Zav-ApoA-I or Zar-ApoA-I might influence these variants in binding Hpt, biotinylated peptides were synthesized (Figure 1). The biotinylated peptides were separately incubated in Hpt-coated wells in ELISAs using avidin-HRP to detect binding. Significant differences in Hpt binding were found for the three peptides. Hpt binding efficiency was in the order Zar-Pep>P2a>Zav-pep (Figure 2). This result suggests that difference in a single amino acid residue, in position 144 or 159 of the ApoA-I sequence, may play a role in the interaction between ApoA-I and Hpt.

A competition assay was performed to rule out the possibility that Zav-pep and Zar-pep bind Hpt on domains different from that recognized by P2a or native ApoA-I. Hpt binding to biotinylated Zav-pep and Zar-pep was evaluated in the presence of increasing amounts of acetylated P2a. The binding of both Zav-pep and Zar-pep decreased with increasing P2a concentration in competition assays (Figure 3). These data suggest that

P2A	Cap-LSPLGEEMRDRARAHVDALRTHLA-$CONH_2$
Zar-ApoA-I	Cap-LSP**R**GEEMRDRARAHVDALRTHLA-$CONH_2$
Zav-ApoA-I	Cap-LSPLGEEMRDRARAHVDA**P**RTHLA-$CONH_2$

Figure 1 Peptide sequences.
Amino acid sequence of the peptides synthesized. Amino acid changes with respect to the ApoA-I sequence are highlighted in bold. The amide peptides were synthesized on PAL-PEG-PS resin (Perseptive Biosystems, Hamburg, Germany) using a peptide synthesizer (348W, Advanced Chemtech, Louisville, KY, USA) and standard Fmoc chemistry (Chan and White, 2000). The N-terminal amino group was acetylated or labeled using *N*-(+)-biotinyl-6-aminoexanoic acid (Fluka, Steinheim, Germany), HATU, and *N*,*N*-diisopropylethylamine (2:2:4 equivalents) in *N*,*N*-dimethylformamide overnight at room temperature. Peptides were cleaved from the resin by treatment with trifluoroacetic acid (TFA)/TIS/water/EDT (90:2.5:2.5:5 by vol.) at room temperature for 3 h. Peptides were purified by HPLC on a Juppiter C18 column (Phenomenex, Torrance, CA, USA) using acetonitrile (0.1% TFA) and water (0.1% TFA) as eluents. Peptide purity and identity were checked by analytical HPLC and ESI mass spectrometry. Cap is an acetyl or biotin group.

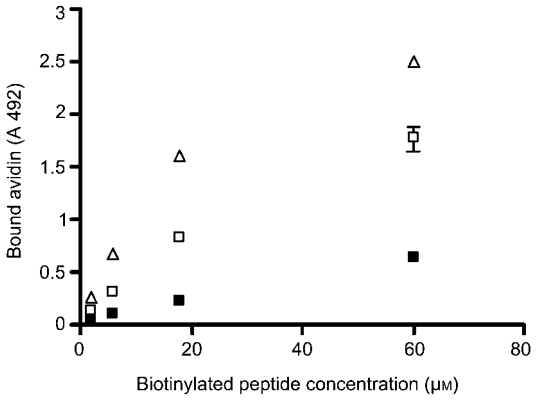

Figure 2 Peptide binding to Hpt.
The binding of peptides (P2a □, Zar-pep △, Zav-pep ■) to Hpt was analyzed by ELISA, essentially as previously reported (Spagnuolo et al., 2005). In particular, microtiter plate wells were coated by incubation of 1 μg of Hpt in 50 μl of 7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3 (pH 9.6). The wells were then loaded with 55 μl of biotinylated peptide (1.8, 6, 18, 60 μM) and after incubation for 90 min at 37°C bound peptides were incubated (1 h at 37°C) with 60 μl of avidin-horseradish peroxidase (HRP) conjugate diluted 1:10 000. The peptide dilution buffer was TBS (130 mM NaCl, 20 mM Tris-HCl, pH 7.3) containing 0.25% bovine serum albumin. The avidin-HRP dilution buffer was TBS containing 0.25% bovine serum albumin and 0.05% Tween. Following each treatment, wells were extensively washed first with TBS containing 0.05% Tween and then with 20 mM Tris-HCl containing 0.5 M NaCl at pH 7.3. The peroxidase reaction was performed in 100 μl of a color development solution (20 mg of *o*-phenylenediamine in 50 ml of 0.1 M sodium phosphate, pH 5, containing 120 μl of 3% H_2O_2). The reaction was stopped by addition of 50 μl of 2.5 M H_2SO_4, and the absorbance at 492 nm was measured. The mean and SEM are reported for three replicates. The program Graph Pad Prism 3 (Graph Pad Software, San Diego, CA, USA) was used to perform *t*-tests.

the Hpt domain involved in interaction with P2a, and therefore with ApoA-I, is also engaged in binding both Zav-pep and Zar-pep.

It was previously observed that ApoA-I can compete with Hb for binding to Hpt (Spagnuolo et al., 2003). It was suggested that steric hindrance of either ligand

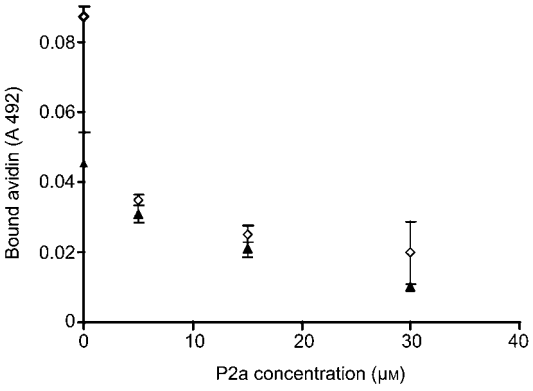

Figure 3 Competition between Zav-pep or Zar-pep and P2a for binding Hpt.
In competition assays to determine Hpt binding, Hpt-coated wells (1 μg) were incubated (90 min, 37°C) with 55 μl of biotinylated Zav-pep or Zar-pep (both 20 μM) in the presence of increasing amounts of acetylated P2a (5, 15, or 30 μM). Bound Zav-pep (▲) or Zar-pep (◇) was detected by incubation (1 h at 37°C) with 60 μl of avidin-HRP diluted 1:10 000. The coating, dilution, washing and color development steps were carried out as described in the legend to Figure 2. Samples were analyzed in triplicate and data are expressed as mean±SEM. The program Graph Pad Prism 3 (Graph Pad Software) was used to obtain trend curves and to perform regression analysis and *t*-tests.

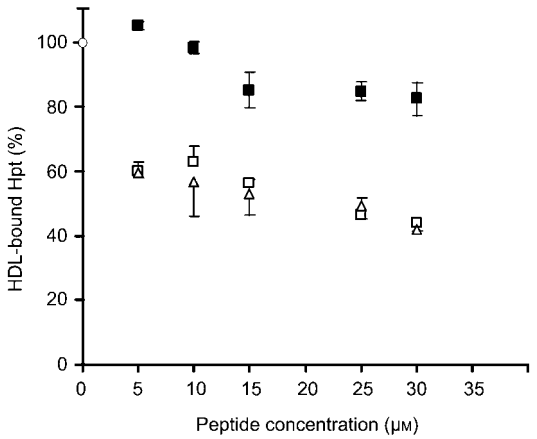

Figure 4 Peptide competition with HDL for Hpt binding.
In competition assays for Hpt binding, wells were coated with 1 μg of HDL. A mixture of Hpt (1 μM) and acetylated P2a (□), Zav-pep (■) or Zar-pep (△) (0, 1, 5, 10, 15, 25 or 30 μM) in CB-TBS buffer (5 mM $CaCl_2$, 0.2% BSA, 130 mM NaCl, 20 mM Tris-HCl, pH 7.3) was incubated for 2 h at 37°C and aliquots of 55 μl were then incubated in the wells for 2 h at 37°C. Hpt binding to HDL was detected using rabbit anti-Hpt IgG and goat anti rabbit-HRP conjugated IgG, as previously reported (Spagnuolo et al., 2005). The amount of immunocomplexes was determined by measuring the absorbance at 492 nm. Data are reported as a percentage of the value obtained for incubation of Hpt alone (◇) and expressed as mean±SEM. Graph Pad Prism 3 (Graph Pad Software) was used to obtain trend curves and perform regression analysis and t-tests.
Acetylated P2a, Zav-pep and Zar-pep were embedded in proteoliposomes (1.5:200:18 peptide/lecithin/cholesterol molar ratio) prepared by the cholate dialysis technique, essentially according to published procedures (Chen and Albers, 1982). In detail, egg lecithin in ethanol was mixed with cholesterol in ethanol in a glass vial. The solvent was evaporated under a nitrogen stream and Tris-saline (85 mM sodium cholate, 150 mM NaCl, 10 mM Tris-HCl, pH 8) was then added to the dried lipids. After vigorous mixing, the micelle suspension was incubated (90 min, 37°C) and repeatedly shaken until it became clear. Then peptide was added to the lipid suspension and further incubated for 1 h at 37°C. The resulting proteoliposome suspension was extensively dialyzed against TBE (140 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.3) at 4°C to remove cholate.
The molarity of Hpt (mixed phenotypes) was expressed as the monomer concentration, that is the unit containing one β subunit (40 kDa) and one α subunit (α_1, 8.9 kDa, or α_2, 16 kDa) (Langlois and Delanghe, 1996; Spagnuolo et al., 2005).

plays a role in competition between ApoA-I and Hb for Hpt. This hypothesis was supported by the finding that P2a does not compete with Hb for Hpt binding (Spagnuolo et al., 2005). Accordingly, Zav-ApoA-I and Zar-ApoA-I were not expected to interfere with Hb binding to Hpt. To test this hypothesis, Hpt (1 μM) was incubated with acetylated P2a, Zav-pep or Zar-pep (20 μM, as liposome-embedded forms) in Hb-coated wells of a microtiter plate according to a published protocol (Spagnuolo et al., 2005). Samples of Hpt alone or Hpt incubated with 20 μM ApoA-I were used as controls. Hpt binding was analyzed using rabbit anti-Hpt antibodies and goat anti-rabbit phosphatase-linked antibodies, using *p*-nitrophenol production for immunocomplex detection. Zav-pep and Zar-pep, similarly to P2a, failed to inhibit Hpt binding to Hb (data not shown). Conversely, ApoA-I competition decreased the amount of Hb-bound Hpt to 40% ($p<0.01$). These results, as expected, indicate that Zav-pep and Zar-pep interact with a Hpt domain other than those involved in capturing Hb.

The peptides were analyzed for their ability to influence Hpt binding to HDL *in vitro*. HDL-coated wells were incubated with Hpt in the presence of different concentrations of liposome-embedded acetylated peptides, using anti-Hpt antibodies to measure the amount of bound Hpt. Zar-pep and P2a displaced up to approximately 60% of Hpt from HDL ($p<0.001$ vs. control with no peptide added), with no significant difference between them (Figure 4). Surprisingly, Zav-pep poorly displaced Hpt from HDL (Figure 4). These results confirm that the amino acid changes differently influence the binding of Zar-pep and Zav-pep to Hpt.

The ability of the peptides to compete with ApoA-I for Hpt binding was also tested in a standard *in vitro* assay of LCAT activity. A pool of plasma treated with dextran sulfate (0.08% in 0.16 M $CaCl_2$) to remove very low-density and low-density lipoprotein, and labeled proteoliposomes (LCAT and ApoA-I-containing [^{3}H]-cholesterol sources, respectively) were incubated with 0.3 μM Hpt in the absence or presence of 0.9 μM P2a, Zar-pep or Zav-pep. LCAT activity was inhibited by Hpt ($p=0.001$), but was restored to different extents when either peptide was present during incubation (Figure 5). In particular, P2a and Zar-pep fully rescued LCAT activity. On the other hand, when Zav-pep was present in the reaction mixture, LCAT activity was not completely restored ($p=0.013$ vs. P2a, $p=0.014$ vs. Zar-pep). The peptides incubated without Hpt did not significantly affect cholesterol esterification (data not shown).

As mentioned above, the influence of ApoA-I mutations, including L159P and L144R, on HDL structure and levels has been investigated. However, a link between specific amino acid changes and LCAT stimulation, HDL

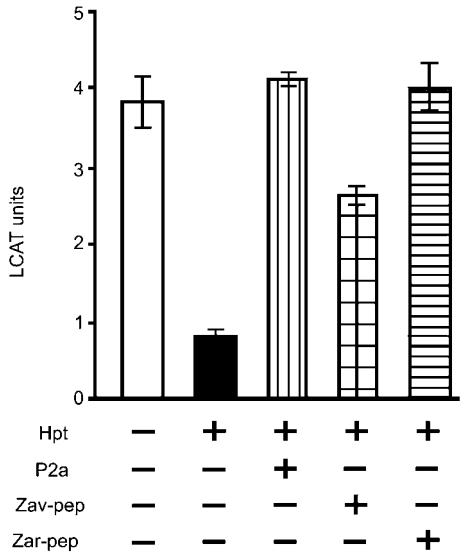

Figure 5 Effect of peptides on the inhibition of LCAT activity by Hpt.
LCAT activity was assayed in the absence or presence of 0.3 μM Hpt and 0.9 μM P2a, Zav-pep or Zar-pep. A pool of plasma samples (treated with 0.08% dextran sulfate, molecular mass 50 kDa, in 0.16 M $CaCl_2$ to remove very low-density lipoprotein and LDL) was used as the LCAT source, and a proteoliposome (ApoA-I/lecithin/[^{3}H]-cholesterol, 1.5:200:18 molar ratio) was used as the substrate. Control assays were performed without Hpt and peptides, or with only Hpt. The proteoliposome was prepared by the cholate dialysis technique (Chen and Albers, 1982) using egg lecithin, [1,2-^{3}H(*N*)]-cholesterol (1 μCi/ml) and unlabelled cholesterol. Subsequent steps were carried out as reported in the legend to Figure 4.
Enzyme activity was measured according to published procedures (Spagnuolo et al., 2005). The LCAT activity is expressed in units corresponding to nmol cholesterol incorporated per hour per ml of plasma. Samples were analyzed in triplicate and data are expressed as mean±SEM.

levels and size, and CAD has not been clearly defined so far. It has recently been proposed that CAD risk is not only associated with low HDL levels (Zannis et al., 2006), as observed in subjects with Zav-ApoA-I (Miller et al., 1998) or Zar-ApoA-I (Recalde et al., 2001). On the other hand, high levels of HDL, which exhibits anti-inflammatory and antioxidant properties that contribute to its atheroprotective role (Rader, 2002), were found also in subjects with documented CAD (Ansell et al., 2003). Therefore, it was proposed that HDL-cholesterol levels do not always correlate with HDL anti-inflammatory function, and we suggest here that the atheroprotective function of HDL or ApoA-I might be influenced by interaction(s) with other proteins, including Hpt, and that such interactions are different for different ApoA-I variants. In this context, we wondered whether the variants Zav-ApoA-I and Zar-ApoA-I, associated with poor cholesterol esterification *in vivo* but normal LCAT activity *in vitro*, might bind Hpt with affinity different from ApoA-I.

The minimal region of ApoA-I required for Hpt binding is restricted to the amino acid sequence L141–A164, which contains helix 6 of the apolipoprotein and overlaps with the LCAT-stimulating region (Lindholm et al., 1998; Sorci-Thomas et al., 1998, 2000). Here we report that two peptides bearing the ApoA-I single-point mutations L159P and L144R, respectively, bind to Hpt with different affinity. Zar-pep displayed binding activity significantly higher than that of P2a and Zav-pep, and displaced Hpt from HDL to a greater extent than Zav-pep. These results are consistent with the full rescue of LCAT activity in the presence of inhibitory Hpt by Zar-pep, whereas only partial LCAT recovery was obtained for Zav-pep. Our data suggest that the role of high levels of Hpt in lowering cholesterol esterification is more effective in subjects with Zar-ApoA-I than in those with Zav-ApoA-I.

It is thought that structural changes strongly affect ApoA-I function in RCT. The ApoA-I sequence of residues 141–164 assumes a helix conformation in the lipid free (Ajees et al., 2006; Davidson and Thompson, 2007) and bound structures (Borhani et al., 1997). Peptides reproducing this region are largely unstructured in aqueous solution, but assume a helix conformation in the presence of micelles (Palgunachari et al., 1996) or when bound to targets. In Zav-pep, the conversion of L159 into P, a helix-breaking residue (as occurring in the Zav-ApoA-I variant), could reduce the length of the helix or cause distortion of the native conformation, which might negatively affect Hpt binding. Conversely, the L144R substitution clearly strengthens the interaction with Hpt, as demonstrated by both experiments on Hpt binding and displacement from HDL with Zar-pep. A change from a hydrophobic (i.e., L) to a charged amino acid (i.e., R) could allow the formation of an extra salt bridge (i, i+3) between R144 and E147, increasing the helix stability and strengthening the interaction of Zar-pep with Hpt. Therefore, our data suggest that low cholesterol esterification in subjects with the Zaragoza variant of ApoA-I (Recalde et al., 2001) might depend on decreased function of the ApoA-I domain that stimulates LCAT, and such function would be negatively affected by Hpt binding. However, it cannot be excluded that *in vivo* both mutations destabilize the lipoprotein particle, preventing normal transport of cholesteryl esters, a feature that might explain the low amounts of these esters observed. In fact, it has been suggested that the strong association between mutations within helices 5–7 and low LCAT activity in plasma is likely due to conformational changes that alter the ability of ApoA-I to stimulate LCAT (Sorci-Thomas and Thomas, 2002). However, the finding of a slightly lower than normal rate of cholesterol esterification in subjects with Zav-ApoA-I (Miller et al., 1998) and Zar-ApoA-I (Recalde et al., 2001) rules out LCAT as a potential cause of the low amount of cholesteryl esters in HDL. Hpt might also display a property different from inhibition of ApoA-I function. During inflammation, this glycoprotein can prevent oxidative damage to bound ApoA-I (Salvatore et al., 2007), thus protecting the LCAT stimulatory domain of ApoA-I against attack by reactive oxygen species. This might explain why ApoA-I mutations display a dominant negative phenotype in heterozygous carriers, as in Zav-ApoA-I (Miller et al., 1998) and Zar-ApoA-I (Miettinen et al., 1997). Whether subjects carrying Zar-ApoA-I are more protected than those with Zav-ApoA-I against oxidative apolipoprotein attack, on the basis of higher binding and shielding by Hpt, is currently a matter of speculation. Further studies are required to assess whether Hpt binding to Zar-ApoA-I and Zav-

ApoA-I influence HDL catabolism, which was suggested to be accelerated in subjects with these variants (Miller et al., 1998; Recalde et al., 2001). At present, it cannot be excluded that the high levels of Hpt present during the acute phase of inflammation influence RCT in these subjects.

In conclusion, a hypothesis on a negative role of Hpt for LCAT regulation in subjects with ApoA-I variants is presented here for the first time. Moreover, our results suggest that Hpt binding assays represent a novel tool for evaluating the effect of ApoA-I mutations on LCAT activity, and might be useful for interpreting the correlation between ApoA-I variants and cholesterol content in HDL.

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