# Mechanism of HDL remodeling induced by CSL112

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## Introduction

CSL112 is apoA-I, the active component of HDL, purified from human plasma and reconstituted to form lipoprotein particles suitable for infusion. It is being developed as a novel means to rapidly stabilize atherosclerotic lesions and reduce the risk of recurrent events after an acute coronary syndrome. CSL112 is designed to optimize cholesterol efflux by the ATP-binding cassette A1 (ABCA1) transporter present in macrophages of atherosclerotic plaques. We have recently demonstrated that infusion of CSL112 into humans and animals causes a strong and immediate increase in capacity of plasma to efflux cholesterol via ABCA1. This elevation is due in part to a rapid remodeling of CSL112 resulting in a very large (>30-fold) increase in plasma levels of lipid-poor apoA-I (pre-β1 HDL), the preferred substrate for ABCA1[1, 2]. In parallel to the rise in lipid-poor apoA-I, CSL112 also causes an increase in particle size of plasma HDL2 and HDL3 subclasses suggesting that CSL112 induces remodeling of plasma lipoproteins.

## Objective

We sought the mechanistic basis for elevation of pre-β1 HDL and ABCA1-dependent cholesterol efflux upon infusion of CSL112.

## Methods and Results

### CSL112 and HDL labeling

CSL112 particles contain two molecules of apoA-I and 110 molecules of phosphatidylcholine[1]. Biotinylation of CSL112 or HDL3 was performed as described by Lund-Katz *et al*[3]. For fluorescence imaging, CSL112 was labeled with DyLight 488 Amine-Reactive Dye (Pierce). The labeling procedure yielded one to two biotin or DyLight 488 molecules per apoA-I molecule in CSL112 or HDL particles.

### *In vitro* incubations, lipoprotein isolation

LDL ($d$ =1.019–1.055 g/mL), HDL2 ($d$ =1.065–1.055 g/mL) and HDL3 ($d$ =1.13–1.21 g/mL) were isolated from cryo-depleted plasma (CSL Behring) by sequential ultracentrifugation[4]. Incubation of CSL112 with individual lipoproteins (1 mg/mL) was carried out in PBS or PBS supplemented with 5% BSA. Three products of HDL remodeling were purified from HDL3 and CSL112 mixtures by sequential ultracentrifugation: large HDL (Fr 1; $d$ =1.12–1.18 g/mL), lipid poor apoA-I (Fr 2; bottom fraction, $d$ =1.225 g/mL), small HDL (Fr 3; $d$ =1.19–1.22 g/mL).
HDL particle size distribution was assessed by 4–30% non-denaturing polyacrylamide gradient gel electrophoresis (NGGE).

### C-efflux

The capacity of the HDL to efflux cholesterol was assessed using [$^3$H]cholesterol-loaded RAW264.7 macrophages. Before initiation of efflux, RAW264.7 cells were loaded with free [$^3$H]cholesterol for 36 h, and stimulated with 0.3 mM 8Br-cAMP for 16 h to up-regulate ABCA1. Efflux was promoted by incubating the [$^3$H]cholesterol-labeled RAW264.7 cells with each individual acceptor for 6 h. Difference in efflux between stimulated and non-stimulated cells is taken as a measure of ABCA1-mediated efflux.

### Studies in human subjects

HDL remodelling was studied in selected healthy subjects receiving infusions of 6.8 g of CSL112 or placebo (n=4 per group) from a completed phase 1 clinical trial (NCT01281774). Plasma samples were obtained at multiple time-points for assessment of apoA-I PK by nephelometry (Pacific Biomarkers) and 2D-gel (3–35% concave gradient) electrophoresis (Boston Heart Diagnostics)[5].

Western blotting of 2D-gradient gels was conducted using polyclonal anti-apoA-I and dilute anti-albumin antibodies. Signal intensities of HDL subfractions were quantified by densitometry and expressed as percentages of the total combined signal measured (100% per blot). HDL subfraction concentrations were then calculated as the measured percentage of the apoA-I concentrations for matching time points.

## CSL112 is rapidly remodeled upon infusion into human subjects

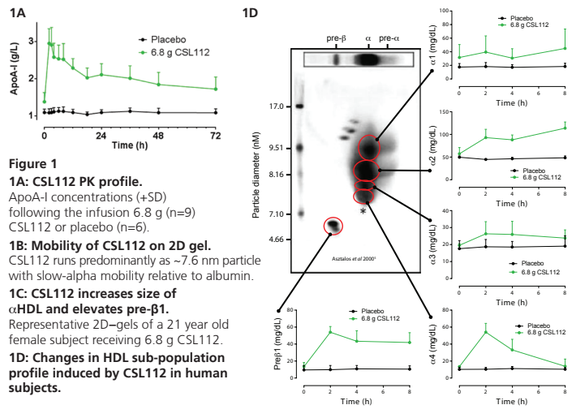


**Figure 1**

**1A: CSL112 PK profile.**
ApoA-I concentrations (+SD) following the infusion 6.8 g (n=9) CSL112 or placebo (n=6).

**1B: Mobility of CSL112 on 2D gel.**
CSL112 runs predominantly as ~7.6 nm particle with slow-alpha mobility relative to albumin.

**1C: CSL112 increases size of αHDL and elevates pre-β1.**
Representative 2D–gels of a 21 year old female subject receiving 6.8 g CSL112.

**1D: Changes in HDL sub-population profile induced by CSL112 in human subjects.**

* indicates position of albumin

- Parent CSL112 is no longer seen in plasma after 2 h.
- Three HDL species become elevated: pre-β1, small (α3/α4) HDL and large (α1/α2) HDL.
- We conclude that apoA-I from CSL112 is incorporated into the endogenous HDL pool.

## Native HDL is sufficient for remodeling of CSL112. Products of the remodeling are efficient acceptors of cholesterol via ABCA1

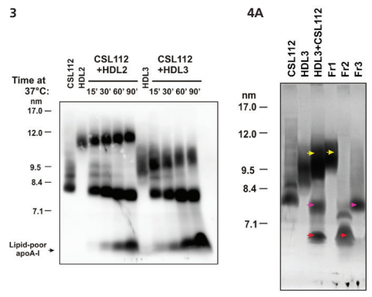


**Figure 3**

**CSL112 can be remodeled by either HDL2 or HDL3.**
CSL112 was incubated with purified HDL2 or HDL3 for the indicated periods of time at 37°C. Samples of individual lipoproteins were included in the analysis as controls. Samples were subjected to NGGE followed by western blotting with anti-apoA-I antibody.

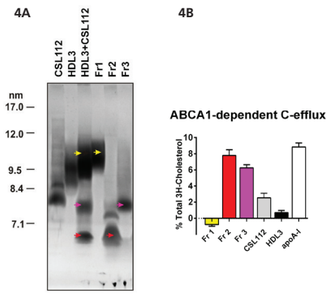


**Figure 4**

**Cholesterol efflux from RAW264.7 cells to individual products of CSL112 and HDL3 remodeling.**
**(A)** CSL112 was incubated with purified HDL3 in PBS for 1 h at 37°C. Products of the remodeling, enlarged HDL (Fr 1), lipid poor apoA-I (Fr 2) and HDL species smaller than parent CSL112 (Fr 3) were purified by sequential ultracentrifugation as described in Methods. HDL particles were separated by NGGE and visualized by Coomassie stain. **(B)** ABCA1-dependent cholesterol efflux from RAW264.7 cells incubated with different cholesterol acceptors at a final concentration of 20 mg/mL. Each fractional efflux value represents the mean ± SD of triplicate measurements.

- Incubation of CSL112 with HDL2 or HDL3 results in the time-dependent disappearance of parent CSL112 with formation of three new species: lipid-poor apoA-I, small HDL3C-like HDL species and enlarged HDL.

- The three products of HDL remodeling can be successfully purified by differential density gradient ultracentrifugation.
- Two small remodeling products, lipid poor apoA-I (Fr2) and small HDL (Fr3), show higher capacity to efflux cholesterol via ABCA1 compared to parent CSL112, native HDL or large HDL (Fr1).

## Remodeling of CSL112 in plasma *ex vivo* resembles that *in vivo*: native lipoproteins are necessary for the remodeling

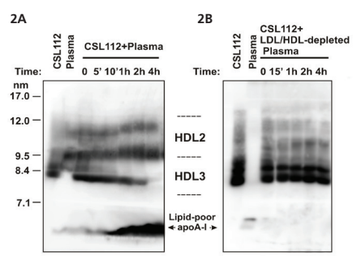


**Figure 2A: In vitro remodeling of CSL112 in plasma is time-dependent.**
CSL112 was incubated with normolipidemic human plasma at a final concentration of 1 mg protein/mL for the indicated periods of time at 37°C. Samples were subjected to NGGE followed by western blotting with anti-apoA-I antibody.

**Figure 2B:** Human plasma was depleted of VLDL/LDL and HDL by ultracentrifugation at density of 1.25 g/mL. The bottom fraction representing lipoprotein-depleted plasma was incubated with CSL112 for the indicated periods of time at 37°C. Samples of CSL112 and plasma alone were included in the analysis as controls. Samples were analyzed as described for Fig.2A.

- Lipoprotein-depleted plasma does not support remodeling of CSL112.
- Native lipoproteins are necessary for the remodeling of CSL112.

- Changes in HDL sub-population profile induced by CSL112 in plasma *ex vivo*:
  - Parent CSL112 is no longer seen in plasma after 2 h.
  - Three HDL species become elevated; lipid-poor apoA-I (pre-β1), small HDL3C-like HDL and large HDL.
  - The average sizes of HDL2 and HDL3 are both elevated.
- All changes are time-dependent and resemble changes seen in human subjects.

## The lipid poor apoA-I produced during remodeling of CSL112 contains apoA-I from both CSL112 and endogenous HDL

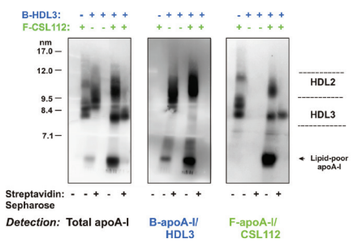


**Figure 5**
Fluorescent-CSL112 (F-CSL112) was incubated with biotinylated-HDL3 (B-HDL3) for 1 h at 37°C or each labeled lipoprotein was incubated alone. Where indicated, the samples were treated with Streptavidin Sepharose beads to remove lipoproteins containing biotinylated apoA-I. Fluorescent apoA-I (F-apoA-I) (right panel) was visualized by fluorescence imaging of the blots; apoA-I (left panel) and biotinylated apoA-I (middle panel) were detected using anti-apoA-I antibody and NeutrAvidin, respectively.

- Lipid-poor apoA-I formed upon incubation of F-CSL112 and B-HDL incorporates biotin and fluorescent labels.
- Fluorescent lipid poor apoA-I is fully precipitated by Streptavidin Sepharose together with biotinylated apoA-I suggesting that lipid poor apoA-I species is a dimer containing one molecule of apoA-I from both F-CSL112 and B-HDL.
- In the presence of plasma, a qualitatively similar pattern of interaction between B-BHDL and F-CSL112 was seen indicating that plasma enzymes do not significantly alter the course of remodeling (data not shown).

## A proposed model of the interaction of CSL112 with HDL

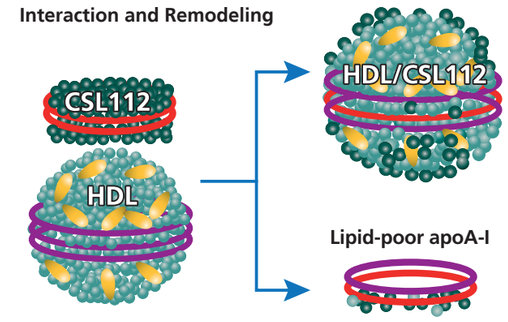


**Figure 6**
CSL112 fuses with HDL to yield a particle with excess apoA-I. A subsequent fission yields a dimeric poor apoA-I species and a larger HDL particle bearing the protein and lipid of both parents. (Model adapted from Rye and Barter 2014[6])

## Conclusions

- CSL112 is remodeled upon infusion into patients or addition to plasma *in vitro* to form lipid-poor apoA-I (pre-β1 HDL).
- Endogenous HDL is necessary and sufficient for the remodeling of CSL112 in plasma. Plasma enzymes do not significantly alter the course of remodeling. Lipid-poor apoA-I formed during this interaction is generated from both CSL112 and HDL.
- We postulate that CSL112 spontaneously fuses with plasma HDL donating protein and lipid components with subsequent release of the lipid-poor apoA-I. Thus the lipid-poor apoA-I is formed by a natural process similar to that which occurs during shrinkage of large HDL particles.
- Products of the HDL remodeling induced by CSL112 show much higher capacity to efflux cholesterol via ABCA1 compared to the original CSL112 and HDL. This finding provides a biochemical basis for the strong elevation of ABCA1-dependent cholesterol efflux and lipid-poor apoA-I observed upon infusion of CSL112 into human subjects.
- These properties make CSL112 a promising candidate for rapidly removing cholesterol from cells in the atherosclerotic plaque.

## References

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## Disclosures

Authors are employees of CSL Behring. Presentation includes investigational uses of drugs.

For presentation at AHA November 18, 2014.