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## **Serum HDL cholesterol uptake capacity in subjects from the MASHAD cohort study: its value in determining the risk of cardiovascular endpoints**

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**Running title:** CUC method and CVD risk factor

**Conflict of interest:** The authors have no conflict of interest to disclose.

## **Abstract**

**Background:** The efficiency of high-density-lipoprotein (HDL) to efflux cholesterol contributes to the reverse cholesterol transport (RCT) pathway as one of HDL's proposed functions, and depends on the ability of HDL to uptake cholesterol. We aimed to investigate cholesterol uptake capacity (CUC) by a newly developed assay in samples from the MASHAD (Mashhad-Stroke and Heart-Atherosclerotic-Disorders) cohort study.

**Method:** The study population comprised 153 individuals developed CVD diagnosed by a specialist cardiologist, over 6-years of follow-up, and 350 subjects without CVD. We used a modified CUC method to evaluate the functionality of HDL in serum samples.

**Result:** The CUC assay was highly reproducible with values for inter- and intra- assay variation of 13.07 and 6.65, respectively. The mean serum CUC was significantly lower in the CVD group compared to control ( $p=0.01$ ). Although, there were no significant differences in serum HDL-C between the groups and there was no significant association with risk of progressive CVD. Multivariate logistic regression analysis showed that there was a significantly negative association between CUC and risk of CVD after adjustment for confounding parameters ( $OR=0.57$ ,  $95\%CI=0.38-0.87$ ,  $P=0.009$ ). The CUC was also inversely and independently associated with the risk of CVD event using Cox-proportional hazards models analysis ( $HR=0.62$ ;  $95\%CI=0.41-0.94$ ,  $P=0.02$ ). We determined the optimum cut-off value of 1.7 a.u for CUC in the population. Furthermore, the CUC value was important in determining the CVD risk stratification derived from data mining analysis.

**Conclusions:** Reduced HDL functionality, as measured by CUC, appears to predict CVD in population sample from north-eastern Iran.

**Keywords:** cholesterol uptake capacity (CUC), HDL function, cardiovascular disease (CVD), cohort study

## **Introduction**

The importance of high-density lipoprotein (HDL) functionality rather than its concentration has been emphasised in the prediction of cardiovascular disease (CVD) (1, 2). Although, many studies have demonstrated inverse associations between serum HDL cholesterol concentrations and CVD risk (1, 3), clinical trials of HDL-raising agents such as niacin and CETP (cholesteryl ester transfer protein) inhibitor have been failed to support the effects of HDL-raising on improved CVD outcomes (4-7). More recent attention has therefore focused on the various functions of HDL including antioxidant, anti-inflammatory, and reverse cholesterol transport (RCT) (8) that may be related to its athero-protective effects. Blood lipids accumulate in tissue macrophages within the arterial intima during atherogenesis, and HDL promotes cholesterol removal from lipid-laden macrophages (9). A measure of the cholesterol-efflux capacity (CEC) has been reported to be inversely related to the incidence of cardiovascular events (10, 11). Returning to the hypothesis that concerns the association between CVD and HDL focused on the concept of the functionality of HDL instead of plasma concentrations, in specific cellular cholesterol efflux (12). As HDL is a pleiotropic particle with a variety of properties, its functions have been difficult to evaluate in human studies. Although the measurement of HDL cholesterol efflux capacity has been exploited in various clinical studies in the case of CVD risk prediction, cell-based assays may have several limitations such as heterogeneity of applied cells, standardization, and experimental variability. The efficiency of HDL to efflux cholesterol from macrophages through the RCT pathway may be an important function of HDL (13) and it depends on the ability of HDL to act as an acceptor of cholesterol. Measurement of cholesterol uptake capacity (CUC) is a newly developed cell-free, sensitive, and high-throughput assay that reflects the functionality of HDL without using radio-isotope labelling and cells (14). Toh et al. (2019) evaluated HDL function through the cell-free CUC method in patients treated with coronary stents, mentioned that impaired HDL function might anticipate stent failure (15). The research has tended to focus on qualitative rather than a quantitative assessment of HDL to improve the prediction of cardiovascular and atherosclerosis clinical outcomes.

Additionally, there have been limited data from cohort study on the association between HDL functionality and CVD risk. Therefore, for the first time, this a cohort study aimed to use a cell-free assay that evaluates CUC and also its optimal threshold to assess HDL function and CVD risk.

## **Materials and Methods**

## **Patient samples**

The MASHAD cohort study (Mashhad Stroke and Heart Atherosclerotic Disorder) is a northeastern population of Iran that started in 2010 with 35 to 65 years old participants without cardiovascular disease (CVD), stroke, and peripheral arterial disease. The follow-up examinations of this study being undertaken every 3 years. Our study comprised three hundred and fifty healthy randomized individuals without clinical cardiovascular disease and 153 subjects without CVD at baseline who then developed CVD outcomes over 6 years of follow-up who were recruited as part of the MASHAD cohort study. The diagnosis of CVD was assessed based on medical interviews, physical examination by two cardiologists using investigations that included: angiography, CT angiography, and ETT over the 6-years duration of follow-up. There is no perfect and complete information about the use of lipid-lowering drugs by patients and healthy individuals. The following anthropometric, demographic, and experimental parameters were measured; fasting blood glucose (FBG), blood pressure measurements (BP), smoking status, and lipid profile (TC, TG, HDL-C, and LDL-C). Informed consent was terminated by all participants and reflected approval by the Ethics Committee of the Mashhad University of Medical Sciences (IR. MUMS. Medical. rec. 1386.250) and the Ethics Committee of the Birjand University of Medical Science (IR. BUMS. rec. 1398.51).

## **Reagents and materials**

The anti-apoA1 antibody (clone311, catalog number MIA1402) and fetal bovine serum (FBS) were purchased from Thermo Scientific. The methyl- $\beta$ -cyclodextrin was purchased from Sigma-Aldrich. BODIPY-cholesterol was purchased from Avanti Polar Lipids. Stock solutions of BODIPY-cholesterol were prepared by dissolving each reagent in DMSO (dimethyl sulfoxide) at 0.5 mmol/L, and the solutions were stored at  $-20^{\circ}\text{C}$ . PBS (Phosphate Buffered Saline) tablets were provided from Sigma-Aldrich that the method of preparation was as follows: dissolving one tablet of PBS buffer in 1 liter of deionized water resulting in 140 mM NaCl, 10 mM phosphate buffer, and 3 mM KCl, pH 7.4 at  $25^{\circ}\text{C}$ . Casein as blocking buffer, Polyethylene glycol (PEG) 6000, and Tris HCl were purchased from Merck. The stock solution of the liposome, as a regular component of the reaction buffer, was made from 12.5 mmol/L cholesterol, 25 mmol/L hydrogenated soy phosphatidylcholine, and 12.5 mmol/L 1,2-dimyristoylsn- glycerol-3-phosphoglycerol.

## **Preparation of the HDL from human serum**

To remove apoB-containing lipoproteins, 100  $\mu$ l of each serum sample was mixed with 20  $\mu$ l of 45% polyethylene glycol (PEG) 6000 in Tris HCL 0.2 M. Then the serum vortex mixing and kept at room temperature for 15 min. The samples were then centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was collected as the HDL fraction (apoB-depleted serum).

### **Cholesterol uptake capacity assay**

We used a modified cholesterol uptake capacity (CUC) method in our study (14). There are three principal steps in this method: (1) preparation of the plate containing the antibody; (2) preparing the serum mixture; (3) cholesterol uptake capacity measurement. At first, 100  $\mu$ l of 5  $\mu$ g/mL of the antiapoA1 antibody (clone 311) in PBS, pH 7.4, was added to each well of the black microplate (96-well). After incubation overnight at 4 °C, the antibody solutions were removed and PBS containing 2% casein solution was added as the blocking buffer and incubated at 37 °C for 2 hours. The blocking buffer was removed and the well washed with PBS two times. Then 10  $\mu$ l of each ApoB-depleted serum sample was incubated with 100  $\mu$ l of 5 $\mu$ M BODIPY-cholesterol in PBS containing 2% BSA and 0.8% liposome stock solution. Followed by shaking microtubes in an incubator (280 rpm, 37 °C) for about 20 hours, 100  $\mu$ L of the apoB-depleted serum mixture was transferred into the wells that were coated by an antibody. The plate was incubated at 37 °C for 3 hours at 350 rpm. Then, the wells were washed with 200  $\mu$ L PBS five times, and 100  $\mu$ L of 20 mmol/L cyclodextrin in PBS was added to enhance the fluorescence signal derived from BODIPY-cholesterol. After the plate was incubated at 25 °C for 30 seconds at 300 rpm, the fluorescence intensity was measured at 535 nm with excitation at 485 nm on the microplate reader.

We expressed the cholesterol uptake capacity as CUC % or the percent of BODIPY-cholesterol uptake by HDL (arbitrary unit or a.u was defined for CUC value). This parameter was calculated by subtracting the background signal from the BODIPY-cholesterol uptake detected for the apoB-depleted serum samples after washing, divided by BODIPY-cholesterol signals for the same at zero-time. Also, the CUC value of each sample was normalized with its HDL-C concentration.

### **Statistics analysis**

SPSS version 20 (IBM Corp, 2011), MedCalc Statistical Software version 16.8 (Bvba, Belgium), and SAS software (JMP Pro 13) were used in this study. Assessment of normality parameters was used by Kolmogorov-Smirnov (K-S) test. The T-test for normally distributed parameters and chi-square for categorical one was utilized to assess the relationship between

the baseline features of individuals in the studied populations. Univariate and multivariate logistic regression analyses was used to examine the association of the CUC and the risk of CVD in the baseline serum samples. P values were considered statistically significant if less than 0.05 ( $<0.05$ ). Moreover, we determined the strength of our study by odds ratio (OR) with a confidence interval (CIs) of 95%. MedCalc Statistical Software was used to compare baseline serum CUC changes (higher and lower than cut-off) in serum samples until the development of clinical CVD was undertaken using Cox proportional hazards models as predictors of clinical CVD.

Data mining analysis was performed using JMP Pro 13 (SAS software). We determined the Log-value which is equal to  $-\log$  (p-value from the Chi-square) of the studied cardiometabolic risk factors to assigning the CVD risk stratification. A nominal logistic fit for CVD was performed to show the efficient characteristics in the model. A dividing system using the decisions tree approach, a multivariate technique was used for both data exploration and prediction, was used to predictive and specialized modelling outputs a flowchart-like structure. The model was divided into two groups at 5 split layers to produce the most division into five subgroups that were as possible for the CVD risk. Scatterplot Matrix was depicted for determining the correlation of lipid profile with cholesterol uptake capacity using data mining analysis.

## **Result**

### **Clinical characteristics of the population**

The study population sample included 153 individuals without CVD at baseline who then developed CVD over 6 years of follow up (CVD group) (76 females (28.4%), aged  $53.79 \pm 6.9$  yrs.) and 350 samples without CVD (192 females (71.6%), aged  $48.9 \pm 7.8$  yrs.). The clinical characteristics and lipid profile of the participants at the baseline of the MASHAD study cohort have been summarized in Table 1.

### **Relationship of cholesterol uptake capacity with the risk of incident CVD**

We evaluated the association between cholesterol uptake capacity (CUC) with cardiovascular event risk by measuring the fluorescence intensity of the labelled cholesterol. The parameter was determined to investigate HDL functionality in serum samples including the percent of BODIPY-cholesterol uptake by apoB depleted sample (CUC %) that was normalized with HDL-C concentration. The mean and range of the CUC value in the MASHAD study

population were equal to 1.18 (0.11-3.4). Our analysis showed that there was a significant difference in CUC values ( $p=0.01$ ) between the group with and without incident CVD events. In contrast, no significant difference in HDL-C was found between these groups (figure 1). As table 2 shows, the mean value of uptake capacity was lower in the CVD group ( $1.07\pm0.47$ ) compared to healthy individuals ( $1.21\pm0.62$ ),  $p=0.01$ .

Subsequently, we examined the association between HDL-C and cholesterol uptake capacity and risk of CVD events developed after 6-years follow-up. According to our result, serum HDL-C was not significantly associated with the risk of progressive CVD using either unadjusted and adjusted models, while a significantly negative association was found between CUC serum samples and risk of CVD outcome in the MASHAD study cohort (OR=0.64, 95%CI= 0.45–0.908,  $P=0.012$ ). Multivariate logistic regression analysis showed that there was a 38% reduction in CVD risk among individuals with a higher CUC after adjustment for confounding parameters including age, sex, BMI, smoking, TG, LDL, TC, diabetes, and hypertension history (OR=0.57, 95%CI= 0.38–0.87,  $P=0.009$ ). Furthermore, the cholesterol uptake capacity measurement was exhibited high reproducibility; the inter- and intra- assay of the CUC criterion were detected as 13.07 and 6.65, respectively.

In the Cox proportional-hazards models (Figure2), there was a significant association of higher cut-off point for CUC and decrease cardiovascular endpoints (Hazard ratio, 0.64; 95% CI, 0.45-0.90,  $p=0.01$ ). Interestingly, after adjusted traditional risk factors for cardiovascular disease, the result showed that cholesterol uptake capacity was independently and inversely associated with the incidence of cardiovascular events (HR, 0.62; 95% CI, 0.41-0.94,  $P=0.02$ ). Kaplan–Meier curve was shown for the CUC value in Figure 2. Our analysis showed that those with serum levels lower than 1.7 a.u. had a higher risk of cardiovascular disease compared to those with higher scores during a 6-years follow-up.

### **Data mining analysis**

In line with these evaluations, we use discriminate analysis to explore the potential markers that are involved in cardiovascular risk assessments (Table 3). According to data mining analysis, age and hypertension history were the most important factors in determining the risk of cardiovascular disease. As the third parameter, the CUC, a measure of HDL functionality, was an important factor in assigning the risk of CVD. The effects of CUC and HDL-C level simultaneously and diabetic history also affect the risk of a CVD event. It can be seen from the data in table 3 that no significant relationship was found between the other parameters



and cardiovascular disease. A major finding is that applying data mining techniques using this kind of data is viable, and determining CUC is of potential clinical value.

We determined the optimum cut-off point of 1.7 a.u. for cholesterol uptake capacity in the MASHAD cohort population using a decision tree derived from data mining analysis. The decision tree with 5 layers, identified the various risk factors for CVD was shown in Figure 3. Based on our results, in the subgroup with  $HDL \geq 35.3$  and  $TG > 115$ , ninety-one percent of subjects were classified in the control group (red strip). In a subgroup with  $HDL < 35.3$ , individuals with  $HDL < 23$ , and  $CUC < 2.37$ , eighty eight percent of individuals were in the case group (blue strip). Meanwhile, the number of the cases was the same as the number of the control classes in individuals with  $HDL \geq 23$ ,  $CUC < 1.7$ , and  $LDL \geq 129.5$ . Finally, in a subgroup with  $HDL \geq 23$  and  $CUC \geq 1.7$ , 93% subjects were in the control classes. A scatter plot matrix is shown in figure 4, and helps the visualisation of the relationships between each pair of response variables like lipid profile and uptake capacity. The narrowness of the ellipse reflects the degree of correlation between variables. As indicated, the ellipse of CUC as a marker of HDL function and HDL-C level was narrow and diagonally oriented, therefore the variables are correlated. But there was no correlation between the CUC and other elements of the lipid profile, since the ellipse was fairly round and was not diagonally oriented when looking at these other parameters.

## **Discussion**

We aimed to assess the potential value of one measure of HDL functionality in new diagnostic procedures for cardiovascular disease in the clinical setting rather than HDL concentration according to the last literature (16). Assessing HDL function with current cell-based assays has some limitations for routine use in the clinic (17). We developed a cell-free assay to investigate the capability of HDL to uptake cholesterol in this study. Our findings demonstrated a significant independently negative association between HDL functionality and the risk of CVD in our cohort study. These results match those observed in earlier studies that have been reported in a cohort study indicated that cholesterol efflux capacity (CEC) as a function of HDL was independent of HDL-C levels associated with a predictor of coronary artery disease (CAD) (11, 18). Among several specified functions of HDL, measurement of the CEC that is attributed to the mechanism of RCT is the most important method to investigate the anti-atherosclerotic effects of HDL (19). Previous studies have hypothesized that the capability of cholesterol efflux of HDL depends essentially on the ability of HDL to uptake extra cholesterol from peripheral tissue (20).

We demonstrate that the mean value of cholesterol uptake capacity (CUC) was lower in the CVD group compared to healthy individuals. Thus, it may be suggested that decreased CUC as an index of impaired HDL functionality, might be associated with a future cardiovascular disease event. This finding supports the first research by Harada et al. (2017) into the study of the CUC on 156 subjects which links the cholesterol uptake capacity as a cell-free assay and recurrence of coronary lesions. It had been demonstrated that among common risk factors of CVD, only CUC remained significantly associated in patients with a controlled LDL concentration (14). Their results were in agreement with the findings of the last study, in which the logistic regression analysis showed that reduced CUC was independently related to neo-atherosclerosis (OR, 0.79;  $P < 0.001$ ) and organization of target lesion revascularization (OR, 0.88;  $P = 0.003$ ). According to the study by Toh, there was a significant correlation between HDL particle concentration and CUC that was measured using NMR (nuclear magnetic resonance spectroscopy). He found that large HDL particles had more association with CUC than small ones (18).

A large and growing body of cohort studies has investigated the cholesterol efflux capacity of HDL using ABCA1 up-regulated cells (10, 11, 21). A single cohort study exists in which Nagano et al. have described the importance of HDL functionality for targeting coronary lesion revascularization within patients who were treated with coronary stents using a cell-free assay (15). Their result demonstrates that CUC at follow-up optical coherence tomography (OCT) was significantly lower in a group who developed neoatherosclerosis (NA+) compared to NA- group (Log Rank  $P < 0.001$ ). To our knowledge, our investigation is the first to undertake the potential relation between CUC as a marker of HDL functionality and hazard ratio of CVD risk in a large prospective cohort study. According to Cox proportional-hazards models, those with serum levels lower than 1.7 a.u. have a higher risk of cardiovascular disease compared to those with higher scores during a 6-years follow-up (Log Rank  $p < 0.003$ ). It seems to be suggesting CUC  $< 1.7$  a.u. is related to the impaired HDL function to uptake cholesterol and exposed to the prominent risk of CVD outcomes in a population-based cohort.

Surprisingly, another important finding, reported for the first time, was that the CUC value was the third most important parameter in assessing the CVD risk stratification, derived using data mining analysis. The evidence from the decision tree suggests that in the subgroup with  $HDL \geq 23$ , 26% of subjects in the CVD group possessed  $CUC < 1.7$ , while in this subgroup 6.7% of individuals with CVD had  $CUC \geq 1.7$ . Some of the issues emerging from this finding

relate specifically to low CUC value and more CVD risk assessment. These findings have also argued that not only HDL concentration but also HDL functionality is considered as a potent risk factor for CVD. HDL metabolism may explain the inadequate effect of HDL particles on CVD risk stratification. As noted by Hirano (1997), the mutation of cholesterol ester transfer protein (CETP), which promotes RCT and results in an increase in HDL-C, does not decrease cardiovascular events (22).

Finally, several limitations may need to be considered in our study. First, the sample size was small compared to the total MASHAD cohort study. The second was the lack of apolipoprotein-A1 measurement that can be used for the normalization of CUC. Thirdly, HDL functionality is a clinically important factor that is determined by environmental and genetic factors that can result in the progression of atherosclerosis and cardio-metabolic disorders. Fourthly, there is no perfect and complete information about the use of lipid-lowering drugs by individuals since they might have an influence on CUC. Therefore, more research needs to be undertaken to explore the association between CUC and cardiovascular risk.

### **Conclusion:**

This project was undertaken to design and evaluate HDL functionality assessment in the MASHAD cohort study by measurement of cholesterol uptake capacity of HDL. Our findings suggest that a low uptake capacity or impaired HDL functionality can be used as a potential biomarker for the prediction of progressive CVD. Also, we showed that cholesterol uptake capacity was independently reverse associated with the prevalence of cardiovascular events. The CUC assay is a high-throughput, high reproducibility, cell-free assay could be used for the assessment of CVD risk in clinical settings.

### **Key funding**

- (1) A decreased CUC value was independently reverse associated with the CVD event
- (2) The mean value of CUC was lower in the CVD group compared to healthy individuals
- (3) Neither HDL-C were not significantly associated with the risk of progressing CVD, while a significantly negative association was found between CUC at the baseline samples and risk of clinical CVD

(4) The CUC value was the third parameter that is significantly effective in assigning the CVD risk stratification derived data mining analysis.

## **Declarations**

### **Ethics approval and consent to participate**

Informed consent was obtained from all subjects using protocols approved by Ethics Committee of the Mashhad University of Medical Sciences (IR. MUMS. Medical. rec. 1386.250) and the Ethics Committee of the Birjand University of Medical Science (IR.bums.rec.1398.51).

### **Consent to publish**

Not applicable

### **Availability of data and materials**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### **Competing interests**

The authors declare no conflict of interests.

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### **Authors' Contributions**

We declare that we contributed significantly towards the research study; MA, TK, EMM, MGM and AA designed the experiments and revised the manuscript. MA, MS, AS and SS performed the experiments. MA and SS wrote the manuscript. MA, HE, AM carried out the data analysis. All authors reviewed, considered and approved the manuscript.

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