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Review

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Assessment of HDL Cholesterol Removal Capacity: Toward Clinical Application

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While there is a controversy regarding the causal relationship between high-density lipoprotein cholesterol (HDL-C) and cardiovascular disease (CVD), recent studies have demonstrated that the cholesterol efflux capacity (CEC) of HDL is associated with the incidence of CVD. However, there are several limitations to current assays of CEC. First, CEC measurements are not instantly applicable in clinical settings, because CEC assay methods require radiolabeled cholesterol and cultured cells, and these procedures are time consuming. Second, techniques to measure CEC are not standardized. Third, the condition of endogenous cholesterol donors would not be accounted for in the CEC assays. Recently, we established a simple, high-throughput, cell-free assay system to evaluate the capacity of HDL to accept additional cholesterol, which is herein referred to as "cholesterol uptake capacity (CUC)". We demonstrated that CUC represents a residual cardiovascular risk in patients with optimal low-density lipoprotein cholesterol control independently of traditional risk factors, including HDL-C. Establishing reproducible approaches for the cholesterol removal capacity of HDL is required to validate the impact of dysfunctional HDL on cardiovascular risk stratification in the "real world".

Key words: **High-density lipoprotein (HDL), Cardiovascular disease, HDL cholesterol (HDL-C), Cholesterol efflux capacity (CEC), Cholesterol uptake capacity (CUC)**

Introduction

Accumulated epidemiological evidence has demonstrated that low levels of circulating high-density lipoprotein cholesterol (HDL-C) are associated with an increased risk of cardiovascular disease (CVD)[1-3]. However, there is a controversy regarding the causal relationship between HDL-C and CVD[4, 5]. In the AIM-HIGH study, the addition of niacin to statin therapy did not reduce cardiovascular events, despite significant improvements in HDL-C levels[6]. Until recently, trials of cholesterol ester transfer protein (CETP) inhibitors, which have been developed to raise HDL-C levels, have failed to provide evidence of cardiovascular benefit[7-9]. Finally, the REVEAL trial has demonstrated that the CETP inhibitor anacetrapib reduced cardiovascular events in patients undergoing statin therapy[10]. However, anacetrapib might work by lowering low-density lipoprotein cholesterol (LDL-C), rather than by increasing HDL-C[11]. Genetic polymorphisms that are associated with increased HDL-C also do not predict a reduced CVD risk[12]. Strikingly, the Framingham Offspring Study has demonstrated that an isolated low HDL-C level does not predict risk when LDL-C and triglyceride (TG) levels are completely normal[13]. Moreover, recent reports have demonstrated that extremely high HDL-C was associated with increased rather than decreased mortality[14, 15].

The limitation of HDL-C as a cardiovascular risk is attributable to the fact that HDL-C is anything more than a snapshot of HDL metabolism. For example, the mutation of CETP, which promotes reverse cholesterol transport (RCT) by transferring a cholesterol ester from HDL to the apolipoprotein B (apoB)-containing lipoproteins in exchange for TG, results in an elevation in HDL-C, but does not necessarily reduce cardiovascular events[16]. The mutation of scavenger receptor class B type I (SR-BI), an HDL receptor expressed abundantly in the liver, also increases

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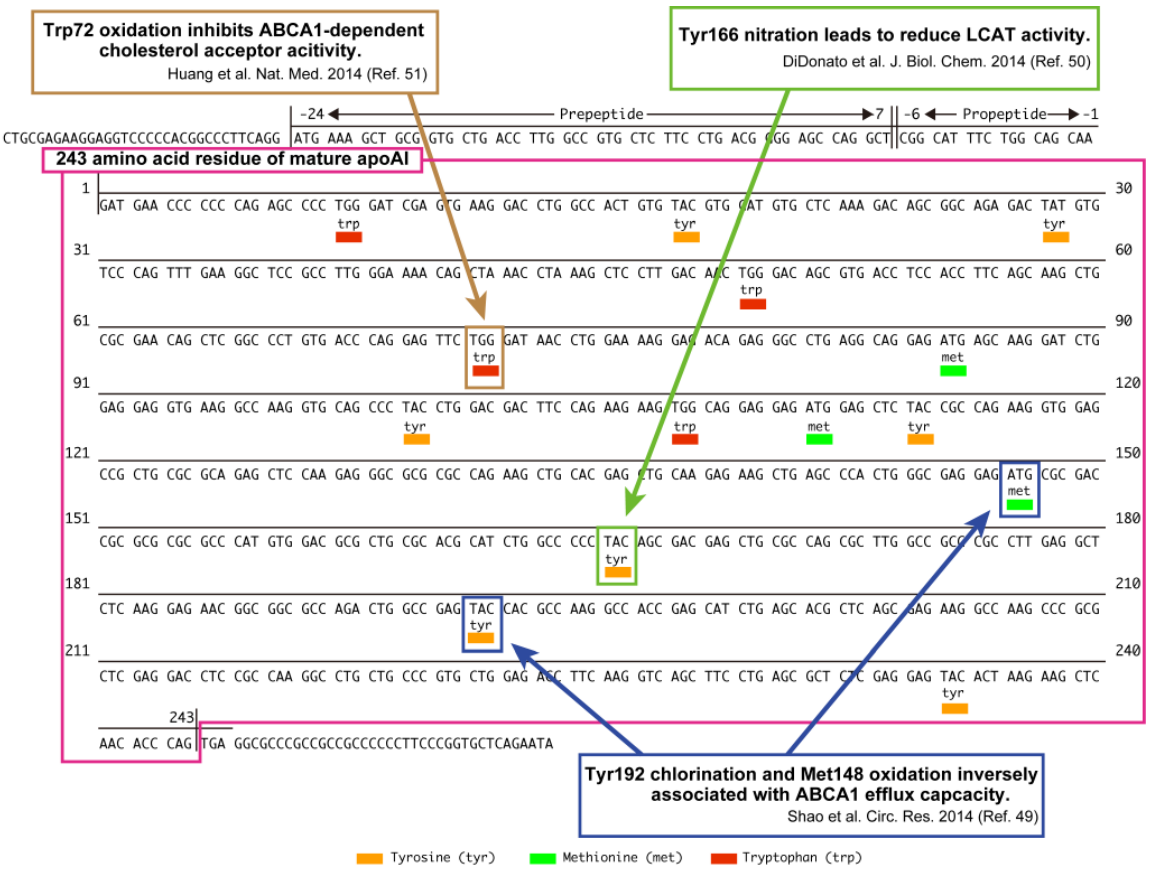

Fig. 1. Post-transcriptional modification of apoAI and CEC

HDL-C, but also accelerates atherosclerosis[17].

On the contrary, recent large cohort studies have demonstrated that the cellular cholesterol efflux capacity (CEC) of HDL, a dynamic rate of initial step in RCT, is associated with both the prevalence and the incidence of CVD, and is a better predictor than steady-state circulating HDL-C levels[18-20]. However, at present, CEC measurements are not instantly applicable as a high-throughput assay in clinical settings. In this review, we discuss the limitations of current CEC assays and future directions.

Cellular Cholesterol Efflux

As most types of cells are unable to catabolize cholesterol, RCT is indispensable for homeostasis. The efflux of cholesterol from cells to serum is an initial step in the RCT pathway. HDL is the component of serum responsible for mediating cholesterol efflux[21]. Cholesterol-enriched macrophages can release cholesterol to HDL using several pathways[22].

First, ATP-binding cassette transporter A1 (ABCA1) is an important player in HDL biogenesis, especially in the setting of cholesterol enrichment. ABCA1 mediates the cellular binding of apoAI, the major structural protein of HDL, and the unidirectional export of cholesterol and phospholipids (PLs) to lipid-free/-poor apoAI, leading to nascent HDL formation[23-25].

On the other hand, SR-BI and ATP-binding cassette transporter G1 (ABCG1) contribute to the maturation of HDL. SR-BI mediates bidirectional cholesterol exchange[22, 26]. ABCG1 does not mediate the cellular binding of HDL, but promotes the transport of free cholesterol from the cell interior to the plasma membrane[27]. The cholesterol translocated by ABCG1 in the cell membrane is exported to HDL via an aqueous diffusion pathway[22]. Lecithin: cholesterol acyltransferase (LCAT) on HDL prevents the return of accepted cholesterol to the cell by esterification, because cholesteryl esters are more hydrophobic than free cholesterol and move to the core of HDL[28]. Mature HDL particles can be remodeled to smaller particles with the release of apoAI by the actions of hepatic lipase, endothelial lipase, and phospholipase A2, which hydrolyze TG and PLs in HDL[29-36].

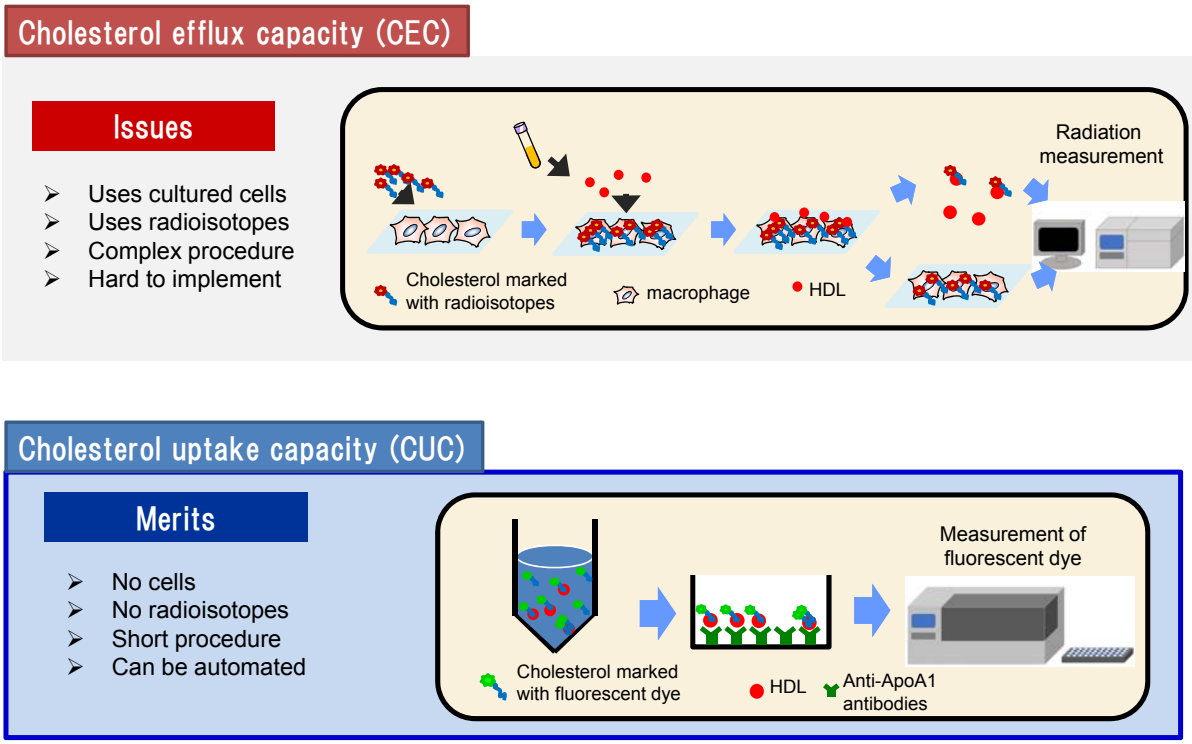

Fig. 2. The procedural schema of CEC and CUC

Current Understanding of CEC

HDL is a heterogeneous population of particles, and ranges in size from <7 to >14 nm[37, 38]. Subspecies of HDL are considered to be involved in different pathways of cellular cholesterol efflux[39, 40]. The smallest apoAI-containing HDL (pre-β-1) and small, dense HDL subfractions (HDL3b and 3c) are principal transporters via the ABCA1 pathway[38, 41]. On the other hand, PL-rich HDL2 is an efficient acceptor of cholesterol because it provides a larger target for effective collisions with diffusing free cholesterol[42-44]. The fatty acid composition of PLs in HDL has also been proposed to affect CEC. We have demonstrated that the oral administration of eicosapentaenoic acid (EPA) improved CEC in patients with dyslipidemia[45], and confirmed the beneficial effects of EPA-rich HDL on cholesterol efflux using reconstituted HDL[46]. Increases in unsaturated PL acyl chains in HDL particles have been demonstrated to result in more efficient cholesterol acceptors[47]. Omega 3 fatty acids have also been shown to increase LCAT activity[48].

On the other hand, the oxidation of several specific amino acids in apoA1 could result in a reduction in CEC[49-51](Fig. 1). Granular leukocyte-derived myeloperoxidase (MPO) promotes the oxidation of apoAI, while paraoxonase 1 (PON1) has antioxidant properties for HDL[52, 53]. We have demonstrated that patients with high MPO levels and low PON1 activity in serum exhibit impaired CEC[54].

Limitations of Conventional CEC Assays

Fig. 2 shows the conventional procedure for a CEC assay. Macrophages are exposed by radiolabeled cholesterol. Then, they are stimulated by HDL, and the excreted cholesterol is measured by a scintillation counter[55].

Although CEC has been used as a marker for CVD, there are several limitations to current CEC assays for clinical application[56]. First, this method requires radiolabeling and cultured cells, and these procedures are time consuming. Second, procedures for CEC measurement are not standardized, which makes it difficult to compare different CEC studies. Varied systems to measure CEC exist, as shown in Fig. 3[56, 57]. Several donor cells are employed for CEC measurements. Fu5AH rat hepatoma cells, which express high levels of SR-BI but lack functional ABCA1, are used to assess SR-BI-mediated efflux[58]. On the other hand, J774 mouse macrophage cells express low levels of SR-BI, and stimulation with cyclic adenosine monophosphate (cAMP) can upregulate its ABCA1 expression[59, 60]. In the Dallas Heart

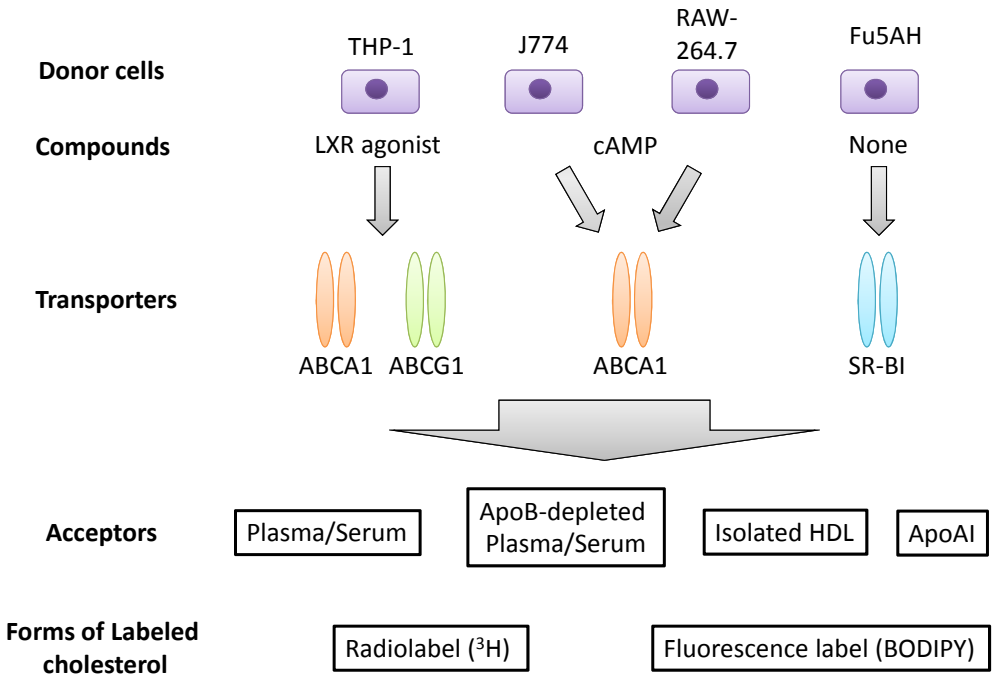

Fig. 3. Varied systems to measure CEC

Adapted from Ref. 57 (Progress in Lipid Research 2018; 69: 21–32).

Study[19] and the European Prospective Investigation of Cancer (EPIC)-Norfolk study[20], J774 cells treated with cAMP were used to measure CEC. On the contrary, Ogura, *et al.* have recently demonstrated that CEC determined using J774 cells without cAMP treatment was also inversely associated with the presence of atherosclerotic CVD in patients with familial hypercholesterolemia[61]. In cases requiring the assessment of ABCA1-dependent CEC, the basal CEC (without cAMP) is subtracted from the total CEC (with cAMP)[62]. Because the ultracentrifugation procedure for HDL isolation requires several days, most of the recent reports employed apoB-depleted serum as the cholesterol acceptor. However, apoB-depleted serum has been reported to contain not only HDL and apoA1 but also other components, such as albumin, that can accept the cholesterol released from macrophages[63]. Moreover, HDL composition and/or size distribution might vary depending on the apoB depletion methods[64]. Li, *et al.* even reported that cholesterol efflux to apoB-depleted serum was paradoxically associated with an increased prospective risk of CVD[63]. While a protocol using radiolabeled cholesterol does not lend itself to the development of a high-throughput assay, fluorescence-labeled cholesterol is alternatively available for CEC measurements. Fractional efflux rates obtained with BODIPY-cholesterol were reported to be greater than those with tritium-labeled cholesterol[65].

The third limitation is that the status of endogenous cholesterol donors would not be accounted for in *in vitro* CEC assays. Changes of *in vivo* macrophage cellular function resulting from various conditions have been reported as follows: phenolic acids increased ABCG1 and SR-BI expression[66]; on the other hand, xanthine oxidoreductase suppressed ABCA1 and ABCG1 expression in macrophages[67]; while we have demonstrated that EPA could improve CEC[45, 46], another group has reported that EPA might reduce ABCA1 functionality in macrophages[68].

Curiously, ABCA-1 dependent CEC was reported to be enhanced rather than impaired in patients with high TG levels[69]. In those patients, a reduction in large HDL particles and an increase in pre-β-1 particles were observed. Concomitantly, SR-BI-dependent efflux, which is mediated mainly by large HDL, decreased. On the other hand, accompanied by an increase in pre-β-1 particles, ABCA-1-dependent efflux was also augmented[69]. However, ABCA1-dependent efflux was determined using J744 cells as described above[69]. The lack of the macrophage ability assessment in an individual might cause overestimation.

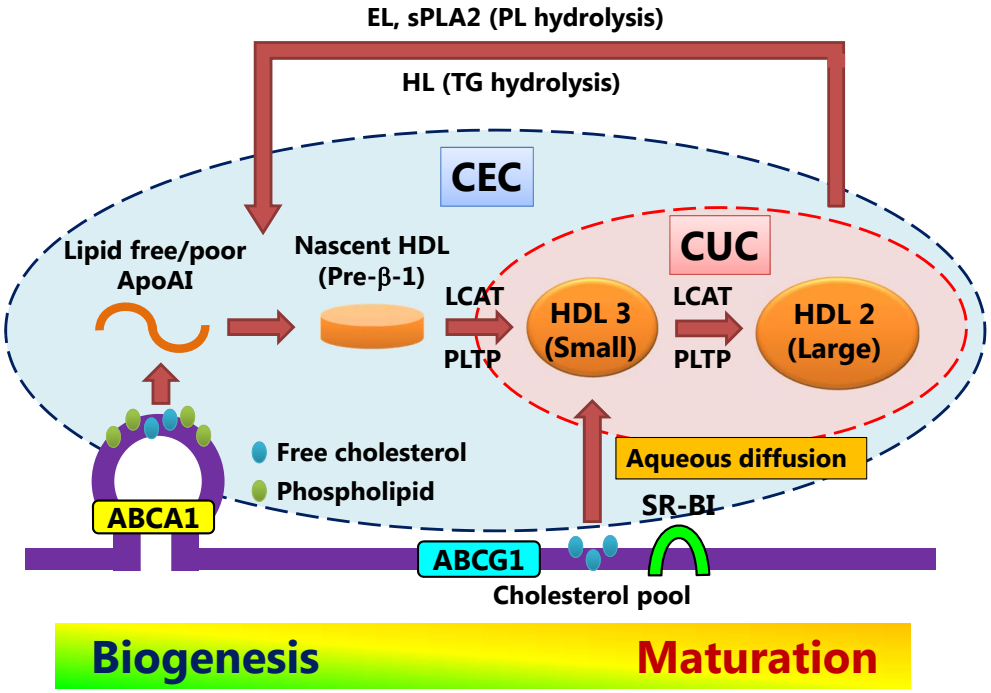

Fig. 4. Differences in concept between CEC and CUC

ABCA1: ATP-binding cassette transporter A1; ABCG1: ATP-binding cassette transporter G1; SR-BI: scavenger receptor class B type I; LCAT: Lecithin:cholesterol acyltransferase; PLTP: phospholipid transfer protein; HL: hepatic lipase; EL: endothelial lipase; sPLA2: secreted phospholipase A2.

Cholesterol Uptake Capacity, A New Measure for HDL Functionality

In order to break through this situation, we have recently established a simple, high-throughput, cell-free assay system to evaluate the "cholesterol uptake capacity (CUC)" as a novel concept for HDL functionality[70].

The procedural schema of our new assay is shown in Fig. 2. After removing apoB, serum is incubated with fluorescence-labeled cholesterol, HDL is captured by specific antibodies for apoAI coated on a microplate, and then the amount of the labeled cholesterol in the HDL is measured using a plate reader. This assay system does not require radiolabeling and cultured cells, and the procedures are simple, with a short turnaround time. Moreover, the application of the anti-apoAI antibody allows a specific evaluation of the ability of HDL to accept cholesterol.

We revealed that CUC was suppressed by MPO treatment, indicating that CUC has the potential to evaluate the oxidation-induced inactivation of HDL[70]. Furthermore, we found that CUC correlated inversely with the requirement for revascularization because of the recurrence of coronary lesions in patients with optimal control of LDL-C. A multivariate analysis adjusted for traditional coronary risk factors, including HDL-C, showed that only CUC remained significant[70].

Difference between CEC and CUC

Since CUC was determined by a cell-free assay, CUC does not reflect ABCA1-mediated efflux (Fig. 4). On the other hand, we demonstrated that CUC was associated with CEC determined using J774 cells without cAMP (non-ABCA1-mediated, basal CEC)[70], which was employed in the study conducted on patients with familial hypercholesterolemia[61]. As the CUC assay is an aqueous diffusion-dependent system, it appears to mainly reflect the contribution of PL-rich, matured HDL to cholesterol efflux (Fig. 4). As expected, HDL particle concentration (HDL-P) measurements using nuclear magnetic resonance spectroscopy demonstrated that large HDL-P showed a more prominent association with CUC than small HDL-P, suggesting that CUC is influenced predominantly by the concentration of matured HDL particles (Fig. 5).

Since the facilitation of HDL biogenesis is a potential therapeutic approach, small HDL particles that promote cholesterol efflux by the ABCA1 path-

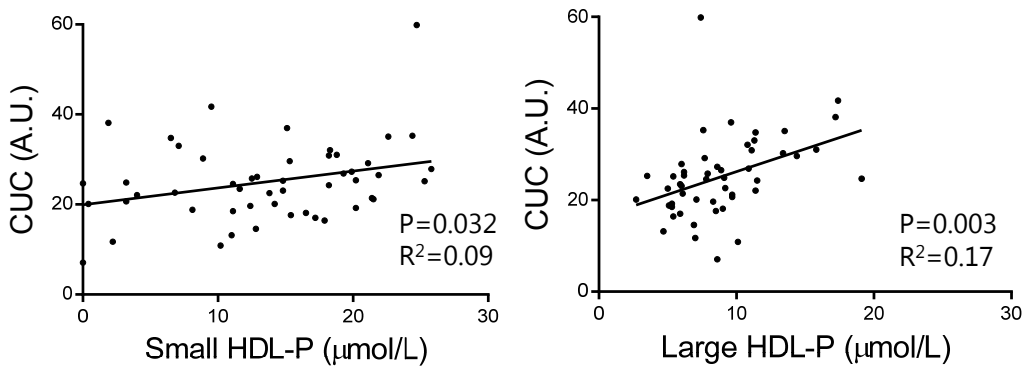

Fig. 5. Correlations between CUC and HDL particle concentration (HDL-P)

Small (diameter range: 7.3–8.2 nm) and large (diameter range: 9.4–14 nm) HDL-P were determined at LipoScience/LabCorp (Burlington, NC) using nuclear magnetic resonance spectroscopy and the LipoProfile-3 algorithm.

way are emphasized as cardioprotective species of HDL[38, 41]. In such a context, CEC was determined using ABCA1-upregulated cells in recent large cohort studies[18-20]. However, all patients with Tangier disease, which is caused by null-mutations in ABCA1, may not necessarily be at high risk of CVD despite marked deficiencies in HDL-C and apoAI[71]. Therefore, the impact of ABCA1-mediated cholesterol efflux on anti-atherosclerotic process is still controversial. On the other hand, most evidence suggests that levels of large HDL particles are inversely related with the risk of CVD, whereas levels of small HDL particles are positively correlated with this risk[72-74]. In dyslipidemic conditions, such as diabetes and insulin resistance, a remodeling of HDL toward smaller particles is assumed to occur due to activity alterations of lipases involved in HDL metabolism and CETP[75, 76]. However, an increase in small HDL particles might seemingly cause an enhancement of CEC, as described above[69]. On the contrary, CUC has the potential utility of cardiovascular risk stratification through the monitoring of disturbances in HDL maturation. Further trials are required to compare the clinical usefulness between "cell-based" CEC and "cell-free" CUC assays.

In terms of the reproducibility of CUC, the intra-assay coefficient of variation (within-run precision) was less than 5% and the inter-assay coefficient of variation (between-run precision) was 7%, both of which are comparable to those of CEC reported in the EPIC-Norfolk study[20]. We are currently developing a completely automated system to measure CUC.

Conclusion

Although CEC as an indicator of HDL function has been used as a marker for CVD, there is no established method for its measurement for use in routine practice. On the other hand, the cell-free assay system to measure CUC will allow a high-throughput characterization of HDL functionality. Establishing reproducible, standardized approaches for HDL-cholesterol removal capacity is required for further validation of its impact on cardiovascular risk stratification in the "real world."

Conflicts of Interest

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