**IL-1 Inhibition and HDL-Containing Fraction Functionality in Patients with Stages 3 to 5 Chronic Kidney Disease**

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**Background:** Systemic inflammation modulates cardiovascular disease risk and functionality of high density lipoprotein (HDL) in the setting of CKD. Whether interventions that modify systemic inflammation can improve HDL function in CKD is unknown.

**Methods:** We conducted a post-hoc analysis of two randomized clinical trials, interleukin-1 (IL-1) trap in patients with CKD stages 3 and 4 (Study A) and IL-1 receptor antagonist (IL1ra) in patients on maintenance hemodialysis (HD) (Study B) to evaluate if IL1 blockade had improved the anti-inflammatory activity (interleukin-6 (IL-6), tumor necrosis factorα (TNFα) and Nod like receptor protein 3 (NLRP3 )), anti-oxidant function (superoxide production) and net cholesterol efflux capacity of HDL. HDL function was measured using lipopolysaccharide stimulated THP-1 macrophages or peritoneal macrophages of apoE-deficient mice exposed to, the apoB-depleted HDL containing fraction obtained from the plasma of the study participants, collected before and after the interventions to block IL-1 effects. ANCOVA was used for between group comparisons.

**Results:** The mean age of the participants was 60 ±13 years old, 72% (n=33) were male, 39% (n=18) were African-American. There were 32 CKD (16 IL-1trap and 16 placebo) and 14 maintenance hemodialysis (7 IL-1ra and 7 placebo) patients. Compared to placebo, IL-1 inhibition, in Study A and B, respectively, reduced cellular expression of TNFα by 15% (p=0.05) and 64% (p=0.02), IL-6 by 38% (p=0.004) and 56% (p=0.08), and Nod like receptor protein 3 (NLRP3) by 16% (p=0.01) and 25% (p=0.02). Compared to placebo, the intervention blunt superoxide production in the treated arm, with the values being higher in the placebo arm by 17% in Study A (p< 0.001) and 12% in Study B (p=0.004). Net cholesterol efflux capacity was not affected by either intervention.

**Conclusion:** IL-1 blockade improves the anti-inflammatory and anti-oxidative properties of HDL fraction in patients with stages 3-5 CKD, including those on maintenance hemodialysis.

**INTRODUCTION**

Systemic inflammation and oxidant stress prevail at all stages of chronic kidney disease (CKD) and are believed to be key mechanisms underlying many adverse consequences of CKD, including cardiovascular disease 1. Anti-inflammatory interventions, specifically anti-cytokine therapies, have been remarkably successful in several chronic diseases such as inflammatory bowel disease, rheumatoid arthritis and psoriasis, and most recently, atherosclerotic cardiovascular disease 2, 3. A landmark study in patients with myocardial infarction demonstrated that administration of a monoclonal antibody targeting interleukin-1 (IL-1) β innate immunity pathway with Canakinumab every 3 months for 4 years led to a significantly lower rate of recurrent cardiovascular events compared to placebo 2. The beneficial effect was observed with no reduction in lipid levels from baseline and hve advanced the “inflammatory hypothesis of atherosclerotic CVD”2. These findings are highly relevant to the CKD population whom suffer from an accelerated atherosclerosis process. We have previously shown that short-term administration of an IL-1 receptor antagonist (IL-1ra) effectively reduced systemic inflammatory markers and increased circulating levels of adiponectin in maintenance hemodialysis patients4, 5. We also reported that an intervention using an IL-1 trap reduced markers of systemic inflammation and vascular oxidative stress and improved endothelial function in patients with stage 3-4 CKD 6. Consideration to inhibit other inflammatory pathway are underway. Thus, the Million Veteran Program, a biobank from the Veterans administration recently revealed that a genetic variant that mimics the effect of an IL-6 blocker was associated with lower risk of CV disease, findings that have prompted randomized trials of IL-6 blockade in CKD 7.

High density lipoprotein (HDL) has a variety of beneficial actions in the general population8-10. In addition to reverse cholesterol transport, whereby HDL transfers cholesterol from the periphery to the liver for excretion, HDL reduces inflammatory processes, limits oxidative stress, inhibits blood clotting mechanisms, and protects the endothelium. Although numerous studies have established that low levels of HDL are associated with increased cardiovascular disease11, 12, recently the emphasis has shifted from circulating levels to functionality of HDL as a better predictor of cardiovascular disease13-15.

CKD impairs many of the protective functions of HDL, including anti-inflammatory and anti-oxidative activities16-21. Non-infectious chronic inflammation is common in CKD1. Whether interventions that reduce systemic inflammation and oxidative stress can improve HDL function in CKD patients is underexplored. In this study, we aimed to determine whether IL-1 inhibition improves the anti-inflammatory and anti-oxidative effects of HDL particles in patients with moderate and severe CKD, including ones on maintenance hemodialysis. We performed a post-hoc analysis from patient samples from two previously completed randomized controlled trials (RCTs) on IL-1 inhibition in CKD stages 3 & 4 (NCT00897715, NCT01663103) and maintenance hemodialysis (HD)2, 22, 23 (NCT00420290) to address these questions.

**MATERIALS AND METHODS**

***Study population and study protocol***

The primary results of the original RCTs have been published previously5, 6. In brief, in Study A, patients with CKD stage 3-4 were recruited at two clinical sites between 2012 and 2014 (University of Colorado Denver Anschutz Medical Campus and Tennessee Valley Healthcare System/Vanderbilt University Medical Center) (NCT00897715 & NCT01663103). IL-1 trap, Rilonacept, an IL-1 decoy receptor that binds IL-1 and neutralizes it before it can bind to cell-surface receptors, was administered subcutaneously, as 160 mg once weekly for 12 weeks after a loading dose of 320 mg6 versus placebo. The primary outcome for this study was changes in endothelial function measured as change in brachial artery flow mediated dilation and the secondary outcome was the effect on hsCRP. The original trial for study A showed that the administration of IL-1 trap in CKD stages 3 &4 improved FMD% by 3.36±2.06% and and reduced hsCRP levels (median [interquartile range]) (baseline: 4.60 [1.90-8.22] mg/L to 12 weeks: 2.16 [0.92-7.38] mg/L. In Study B, maintenance hemodialysis patients were recruited from Tennessee Valley Healthcare System and Vanderbilt University Medical Center between 2008 and 2010 (NCT00420290). Human recombinant IL-1ra, Anakinra (100 mg subcutaneous, Amgen, Thousand Oaks, CA, USA) or placebo was injected at each dialysis session for 4 weeks)5. For study B the primary outcome was the effect of IL-1ra administration in hsCRP. The original trial for study B showed that IL-1ra effectively reduce hsCRP by 50%. Exclusion criteria for both trials were active or history of chronic infection [human immunodeficiency virus (HIV) disease], hepatitis B, hepatitis C, tuberculosis/tuberculin test positive/QuantiFERON TB gold positive, history of malignancy within the past 5 years, hospitalization in the prior month, immunosuppressive medication within the past year, or any investigational drug within 1 month before the study. Presence of a dialysis catheter was an exclusion criterion for the study on maintenance hemodialysis patients. Both studies were approved by the respective Institutional Review Boards. Signed informed consent was obtained from all study subjects. A consort flow chart is included (Figure 1). For study B (maintenance HD), the IL-1ra trial, all 14 participants who completed the original trial were included in this analysis5. For study A (CKD stages 3 & 4), the IL-1trap trial, 16/21 (76%), patients in each arm had sufficient remaining blood samples for analysis for this post-hoc study (baseline characteristics of the parent trial for Study A are presented in Supplemental Table 1) 6.

***Procedures***

***Blood sample collection***

Baseline blood samples were collected during the baseline visit within the 2 days prior to staring the intervention. The intervention was initiated after the baseline samples and measurements were completed during the baseline visit for both studies. For the dialysis study, blood samples were collected before dialysis. End of study blood samples were collected within one week after the last dose of the study drug was administered.

***HDL isolation and assessment of plasma profile***

Blood samples were collected by venipuncture into EDTA tubes, centrifuged at 1700 *g* for 15 min at 4oC. The samples were aliquoted and were stored at -80oC and thawed only once which we and others have shown as having minimal effect on functionality14, 24, 25. The HDL containing fraction used in these studies was obtained after removal of apoB lipoproteins which is the prevailing method in clinical studies examining HDL function. Patient specimen aliquots were treated with PEG solution to precipitate apoB-containing lipoproteins by adding 100 µl of polyethylene glycol (PEG) solution (20% PEG 8000 in 200 mM glycine, pH 7.4) to patient plasma (250 µl). After 15 minute incubation, the samples underwent high-speed centrifugation (1,900g for 15 min at 4°C). The supernatant was then removed and the apolipoprotein B (Apo B)-depleted HDL-enriched fraction 18, 26, 27 used as in previous studies assessing HDL functionality. We chose this method of assessing HDL fraction functionality based on its use in the previous studies and possible confounding effects of alternative approaches relying on density gradient separation, where added substances may alter functionality of HDL28 Plasma levels of total cholesterol, LDL, triglycerides, and HDL were measured enzymatically (Cliniqa, San Marcos, California).

***Macrophage inflammatory reaction with HDL***

HDL modulation of inflammatory effects was measured using the established cytokine response in LPS-activated cells25. Briefly, THP-1 cells (American Type Culture Collection, Manassas, VA) were plated and differentiated using RPMI 1670 containing 10% fetal bovine plasma and 50 ng/ml phorbol 12-myristate 13-acetate. THP-1 macrophages were exposed to apoB-depleted HDL fraction (18 μg cholesterol/ml) and LPS (50 ng/ml) for 4 h. Total RNA was extracted from cells with RNeasy Mini Kit (QIAGEN) as previously described18, 25. Quantification of human interleukin-6 (IL-6), tumor necrosis factor–α (TNF-α), NOD-like receptor protein3 (NLRP3) and endogenous control human beta actin gene expression was performed by real-time reverse transcriptase polymerase chain reaction (PCR) using CFX96TM Real-Time System (BIO-RAD). Probes for IL-6 (Hs99999032\_m1), TNF-alpha (Hs99999043\_m1), NLRP3 (Hs00918082\_m1) and beta actin (Hs01060665\_g1) were obtained from Applied Biosystems (Foster City, Ca). These inflammatory markers were chosen because of their well-established participation in atherogenesis and critical role in the IL-1b inflammatory pathway.2, 22, 23

***Macrophage generation of reactive oxygen species***

Cellular production of superoxide was measured as the formation of a superoxide specific product of dihydroethidium, 2-hydroxyethidium, using high-performance liquid chromatography (HPLC) analysis in THP-1 cells exposed to apoB-depleted HDL fraction as described in the inflammatory response studies29.

***Net cholesterol efflux assay***

Thioglycolate-elicited peritoneal macrophages were isolated from apoE-deficient mice and plated as previously described30. After washing, DMEM with 40ug ac-LDL/ml was added to each well (To) and incubated for 40h. Cells were exposed to 2% apoB-depleted HDL fraction (PEG 8000-precipitation) in DMEM for 24h, then washed, dried, and incubated with isopropanol to extract cellular lipid. To examine the ability of the apoB depleted fraction to mediate the **net efflux** of cholesterol, cellular cholesterol mass was measured as described previously **31**. Fluorescent intensity was measured at excitation wavelength 530 nm and emission wavelength 590 nm. Cellular cholesterol mass was calculated based on standard curves and corrected by protein32. Net cholesterol efflux capacity was calculated as (cholesterol levels in To – cholesterol levels in efflux wells)/ cholesterol levels in To x100%.

***Study endpoints***

The primary outcomes were inflammatory biomarkers (IL-6, TNFα. and NLRP3) response in LPS-stimulated THP-1 macrophages to the patient’s apoB-depleted HDL fraction before and after the intervention for each trial. Secondary outcomes included the change in the production of superoxide in LPS-stimulated THP-1 macrophages and the changes in net cholesterol efflux capacity before and after the intervention for each trial. Exploratory outcomes included any effects on lipid profile. Covariates included demographics, body mass index, diabetes, serum albumin and statin use.

***Statistics***

Data are presented as mean ± SD or as median with interquartile ranges depending on the distribution of the particular variable or as proportions and compared using Mann-Whitney U or χ2 tests when appropriate. Analysis of covariance (ANCOVA) was used to estimate the percent change (regression coefficient from the ANCOVA model) as a function of treatment group from baseline to end of the study for all outcomes, which refers to the difference in percent change between the treatment and the placebo groups33. We did not generate the percent change at an individual level because within patient change is affected strongly by regression to the mean and measurement error rather we selected ANCOVA as recommended by several authors for this setting. ANCOVA has additional advantages including control for baseline differences and incorporation of randomization strata as covariates. Outcome variables were log-transformed to improve normality in residuals, and the baseline value of the outcome variable was adjusted as a covariate. Because of the small number of participants, no adjustment of other variables was performed, as in the parent trials 5, 6. A P value of less than 0.05 was considered to indicate statistical significance.  All reported P values are two-sided. Analyses were performed using STATA version 15.

**RESULTS**

***Baseline characteristics***

There were 32 CKD (16 active drug and 16 placebo) and 14 maintenance hemodialysis (7 active drug and 7 placebo) patients. Baseline characteristics of both CKD and ESRD participants have been described in detail in each of the parent trials5, 6. For CKD patients, the mean age was 65.0±10.3 years, 28% were female (n=9) and 75% were white (n=24). The mean age for maintenance hemodialysis patients was 49.0±13.0 years, 29% were female and 71% were African American. **Table 1** shows baseline characteristics of the two study groups at the time of randomization. Patient enrollment, randomization, and completion flow diagram for both trials is shown in **Figure 1**.

***IL-1 inhibition improves HDL anti-inflammatory cytokine response and of NLRP3 inflammasome expression***

*In study A (CKD stages 3 & 4)* IL-1trap effectively reduced the cellular expression of biomarkers of inflammation and oxidative stress (**Table 2, Figure 2A**). For IL-6, the intervention reduced the cellular IL-6 mRNA expression by 38% (p=0.004) compared to placebo. For TNFα, mRNA expression was reduced by 15 % (p=0.05) compared to placebo, for NLRP3 the intervention reduced the mRNA expression by 16% (p=0.01). *In study B (maintenance hemodialysis)* IL-1ra also reduced the cellular expression of biomarkers of inflammation and oxidative stress. However, statistical significance was not observed for all biomarkers (**Table 2, Figure 2B**). For IL-6, the intervention reduced the cellular mRNA expression by 56% (p=0.08) compared to placebo. For TNFα, mRNA expression was reduced by 64% (p=0.02) compared to placebo, and for NLRP3 (**Table 2, Figure 3**) the intervention reduced the mRNA expression by 25% (p=0.02) compared to placebo.

***IL-1 inhibition improves HDL capacity to decrease cellular reactive oxygen species generation***

To further investigate the effects of IL-1 inhibition on HDL fraction function, we measured superoxide production in LPS-stimulated THP-1 macrophages exposed to HDL fraction before and after the intervention (**Figure 4**). Compared with placebo, IL-1 inhibition led to a significant blunting in reactive oxygen species generation in response to HDL.

In Study A, reactive oxygen species production increased to a lesser extent in the IL-1 trap arm from a median of 490 pmol/mg (IQR 471-520) to a median of 513 pmol/mg (IQR 482-526) at the end of the study, while in the placebo group, reactive oxygen species production increased from a median of 502 pmol/mg (IQR 469-513) to a median of 595 pmol/mg (574-610) at the end of the study. The comparison between groups for the effect of the intervention was statistically significant (p<0.001) with 17% more reactive oxygen species production in the placebo group compared to the intervention group.

In Study B, reactive oxygen species production was also blunted in the IL1ra administration arm from a baseline median of 790 pmol/mg (IQR 766-813) to a median of 816 pmol/mg (IQR 788-840) at the end of the study, while in the placebo group, reactive oxygen species production increased from a baseline median of 780 pmol/mg (IQR 748-782) to a median of 921 pmol/mg (IQR 899-924) at the end of the study. The comparison between groups for the effect of the intervention was statistically significant (p=0.004) with 12% more reactive oxygen species production in the placebo group compared to intervention group. (**Figure 4**).

***IL-1 inhibition and HDL net cholesterol efflux capacity***

There were no significant changes in net cholesterol efflux capacity with either intervention or placebo in either study. In Study A, net cholesterol efflux capacity showed a median of 26% (IQR 25%, 27%) before intervention and 29% (27%, 31%) after intervention and in the placebo group from a median of 32% (IQR 30%, 33%) to a median of 38% (IQR 31%, 43%) (p=0.3). In Study B, there were no significant changes in either group, from a median 38% (IQR 30%48%) to a median 36% (IQR 29%, 47%) and from a median 37% (IQR 29%45%) to a median 38% (IQR 29%,46%), in the intervention and placebo groups, respectively (p=0.5)

***IL-1 inhibition effects on plasma lipids***

There were no significant changes in plasma lipid profiles including LDL, HDL-C and triglycerides in response to IL-1 blockade in study A (CKD stages 3 & 4)**.** In study B (maintenance hemodialysis) there was a decrease in HDL of 15% driven by changes in the placebo arm (p=0.02) (**Table 3**).

**DISCUSSION**

CKD is associated with dysfunctional HDL, which acquires a pro-inflammatory and pro-oxidative phenotype that can promote adverse consequences of CKD, including cardiovascular disease risk17,19, 34-38 . The current study examined whether blockade of IL-1 activity by a direct inhibitor or a receptor antagonist could improve HDL fraction function in patients with CKD stage 3–5, including individuals on maintenance hemodialysis. Compared to HDL of participants who received placebo, HDL fraction of CKD patients who received IL-1trap or IL-1ra had significantly improved cellular anti-inflammatory capacity, reflected by reduced mRNA expression of cytokines and NLRP3. Additionally, there was an amelioration of enhanced oxidant effects of HDL, reflected by reduced superoxide production in LPS-stimulated macrophages of the treated patients. Lipid handling, assessed by net cholesterol efflux capacity of HDL, was not affected by either intervention compared to placebo.

Although multiple epidemiological studies have firmly established the inverse relationship between HDL-C concentration and cardiovascular disease risk, treatments that raise HDL-C concentration have not reduced cardiovascular events 11, 12, 39. These observations have given rise to the new concept that HDL function is a better predictor of risk than HDL concentration21. Although these results have stimulated intense interest in factors affecting HDL functionality13-15, 40, it is currently uncertain which particular HDL functionality or panel of functionalities is most important, and some of the pleiotropic actions of HDL are under intense investigation. Nonetheless, recent reports indicate that beneficial functions of HDL can indeed be restored7, 27, 41, 42. Anti-inflammatory treatment of patients with rheumatoid arthritis with methotrexate and infliximab improved HDL-directed functions in endothelial cells, including nitric oxide bioavailability and superoxide production42.These results are highly relevant to patients with CKD who have consistently demonstrated HDL dysfunction16-21, and have a high prevalence of chronic non-infectious inflammation, which contributes to their CVD risk1, 22, 23, 40. Whether HDL functionality can be improved in this high-risk population is unknown.

To test the hypothesis that IL-1 β blockade can improve HDL functionality in the setting of advanced CKD, the current study used HDL fraction isolated from participants of two randomized controlled trials (stage 3-4 CKD and maintenance hemodialysis)5, 6. IL-1 blockade reduced macrophage expression of IL-6 and TNF-α exposed to the HDL fraction of treated subjects. Thus, despite different levels of kidney function (stage 3-4 CKD and maintenance hemodialysis), different IL-1 inhibitors (IL-1 trap and IL-1ra) and different duration of treatment (12 weeks in CKD and 4 weeks in maintenance hemodialysis participants), IL-1 blockade effectively improved HDL fraction anti-inflammatory actions. In addition, HDL fraction of patients treated with IL-1 blockade also had blunted cellular superoxide production in LPS-exposed macrophages compared to exposure to HDL fraction in the placebo treated group of each study. These results complement our previous observations that anti-inflammatory therapy targeting IL-1β benefits vascular function in patients with CKD stages 3 and 4, including improved brachial artery flow-mediated dilation, an index of impairment of endothelium-dependent dilation, and endothelial cell NADPH oxidase expression6. We have also observed increased levels of adiponectin in both studies suggesting that the metabolic benefits of IL1 blockade is remarkably consistent and broad 4, 5. Since patients with advanced CKD requiring dialysis do not consistently respond to conventional lipid–lowering treatments43, 44, these results suggest the possibility that cytokine-based therapy could represent a novel, complementary, non-lipid lowering intervention to reduce the high cardiovascular disease risk in this vulnerable population.

The mechanisms by which blocking the actions of IL1 leads to improvements in HDL fraction functionality in the setting of advanced CKD are unclear. The current understanding involves a signaling cascade that moves upstream from C-reactive protein (CRP) to IL-6 to IL-123, 40, 45. Our previously reported findings of reduced circulating high-sensitivity CRP and IL-6, together with blunted cellular IL-6 response to HDL with IL-1 inhibitor treatment, fits well with this pathway. Critically, interleukin-1β is controlled by a cytosolic multi-protein complex, the inflammasome, which includes NLRP3. HDL can downregulate NLRP3, which in turn, reduces secretion of IL-1β46. It is therefore possible that the interaction between the inflammasome and HDL is abnormal when HDL is dysfunctional, as is the case in the CKD population. In particular, since activation of NLRP3 depends on production and binding of reactive oxygen species to NLRP3, decreasing reactive oxygen species can inhibit the NLRP3 inflammasome. Our findings that IL-1 blockade significantly reduces cellular reactive oxygen species production by HDL fraction and lowers NLRP3 expression in CKD and maintenance hemodialysis patients are consistent with this possibility. These results reiterate the central role played by the inflammasome and support the growing interest in directly targeting NLRP3 to treat atherosclerosis3, 23, 40, 45, 47.

In contrast to the beneficial effects on HDL’s anti-inflammatory and anti-oxidant capacity, IL-1 blockade did not influence net cholesterol efflux capacity. It is possible that a larger sample size is needed to evaluate if IL-1 trap or IL-1ra improves cholesterol efflux capacity. However, we previously observed this lack of a parallel response between efflux capacity and heightened inflammatory response in maintenance hemodialysis subjects, as well as children with moderate CKD or ESRD requiring dialysis18, 25. Studies in other populations also reiterate that HDL net efflux capacity and other vasoprotective-functions are not necessarily linked42, 48. These observations suggest that anti-inflammatory therapies have greater impact on the vascular functions involving inflammation and oxidative stress than lipid handling functions of HDL. Indeed, several studies have shown HDL-associated oxidant stress markers correlate with clinical outcomes in the CKD population19, 49.

It is worth emphasizing that IL-1 inhibition did not improve the plasma lipid profile in our study in CKD stages 3 & 4. We did observe an effect on HDL in maintenance HD patients (study B) driven by a decrease in the HDL levels in the placebo arm. Notably, however, in the much longer duration CANTOS trial that included 1875 patients with GFR<60 ml/min followed for 48 months of Canakunimab in three different doses there was no effect on plasma lipids (HDL and LDL) 2, 22. However, Canakunimab significantly reduced high-sensitivity C-reactive protein and significantly lowered cardiovascular events compared to placebo, even in patients with CKD, even in the absence of any effects on atherogenic lipids 2, 22. Indeed, systemic inflammation which prevails at all stages of CKD may be highly relevant to the recently advanced “inflammatory hypothesis of atherosclerotic CVD”. 2, 22. These observations represent a departure from the previous focus to increase the levels of circulating HDL to targeting HDL function with metabolic benefits of HDL 7, 27, 41.

HDL has well described benefits to protect the endothelium. In patients with CKD, HDL strongly inhibits nitric oxide production, promotes superoxide production, and reduces HDL capacity to protect endothelial cells against monocyte adhesion molecules 50. Unfortunately, we were not able to test the effect of HDL fraction on these parameters before and after the intervention. Nonetheless, in study A, IL-1 blockade improved endothelial function measured as brachial artery flow-mediated dilation, suggesting a possible role for HDL in this response.6

Our study has several strengths, including the randomized placebo-controlled trial design of the parent studies and drug administration performed under direct supervision. The experiments in this study were all done in a blinded fashion, albeit this was a post-hoc analysis without a specific a priori power analysis. In addition, the effectiveness of IL1 blockade in reducing the systemic inflammatory response paralleled the effectiveness in improving HDL functionality across a spectrum of CKD (i.e., stages 3-5, including individuals on maintenance hemodialysis). Our study also has several limitations. Both parent studies were short-term, mechanistic in nature, and both had relatively small sample sizes consistent with the power calculations needed for the primary outcomes of the parent studies. Accordingly, we were underpowered for some of the measurements and we assessed multiple markers in several pathways generating multiple comparisons. These studies were performed in patients with mild to moderate chronic inflammation and may not be generalizable to patients that are not inflamed. However, inflammation is highly prevalent in both CKD and ESRD patients. The study lacked the ability to examine the association with hard endpoints, such as CV events or mortality, as well as safety parameters such as increased risk of infections and thrombocytopenia that were observed in Canakunimab trial. Notably, we observed two episodes of infection, three injection site reactions, and one episode of thrombocytopenia. Finally, there is no universally accepted “standard method” of HDL isolation or functional assay and the *in vitro* methodology used in our study may not completely recapitulate the *in vivo* effects of disease on HDL composition, metabolism or macrophage function28. However, a number of clinical studies used this approach to assay cholesterol efflux capacity and the anti-inflammatory functions of HDL, and these measures of HDL function have been shown to add independent incremental value in CVD risk prediction models14, 15. Indeed, the apoB-depleted serum fraction is the most often used method in clinical studies to assay cholesterol efflux capacity and anti-inflammatory functions. The efflux assay is based on measuring cell cholesterol mass, which assures that the net flux of cholesterol is assessed, thereby controlling for the influx side (i.e. FC influx, CE selective uptake, HDL uptake, and degradation) that may differ between populations. Nonetheless, the in vitro method has inherent limitations including that it does not precisely reflect the in vivo HDL metabolism or macrophage function.

In conclusion, our results suggest that IL-1 blockade improves HDL-mediated anti-inflammatory and antioxidant function in patients with stage 3-5 CKD, including individuals on maintenance hemodialysis. These findings suggest potential utility and possible mechanisms in antagonizing IL-1 in this population to reduce the atherosclerotic burden and improve CV outcomes in the setting of moderate-to-advanced CKD. Larger studies of longer duration are required to confirm our study findings and evaluate the effects of these interventions in cardiovascular morbidity and mortality in patients with moderate to advance CKD.

**DISCLOSURE**

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Study A: This work was supported by a Department of Veterans Affairs Clinical Science Research & Development (CSR&D) Service Office merit review award “Dysmetabolism of Chronic Kidney Disease and Vascular Health (I01 CX000982-01 A.M.H). The parent studies were supported by career development award “Inflammation in CKD and CVD—the Role of Genetics and IL-1ra.” (2-031-09S for A.M.H), by an American Heart Association post-doctoral fellowship award (12POST11920023 to K.L.N.). The study drug and matching placebo were kindly provided by Regeneron Pharmaceuticals (Tarrytown, NY).

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Table 1 Baseline characteristics.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variable** | Study A  CKD stages 3 & 4  (n=16) | | Study B  Maintenance hemodialysis | |
| IL-1 trap (n=16) | Placebo (n=16) | IL-1ra (n=7) | Placebo (n=7) |
| ***Demographics*** |  |  |  |  |
| Male sex—% (n) | 69% (11) | 75% (12) | 71% (5) | 71% (5) |
| Age, yr (mean+SD) | 62+12 | 67+8 | 50.6 ± 14 | 47.7 ± 12 |
| Race, % (n) |  |  |  |  |
| African American | 25% (4) | 25% (4) | 86%(6) | 57%(4) |
| ***Clinical Characteristics*** |  |  |  |  |
| Statin, % (n) | 50% (8) | 75% (12) | NA | NA |
| Diabetes | 50% (8) | 56.25 (9) | 0% (0) | 43%(3) |
| BMI, kg/m2 (mean+SD) | 32+6.2 | 31+5 | 34±6 | 29±10 |
| ***Inflammatory Biomarkers*** |  |  |  |  |
| Serum albumin (g/dL) | 4.0 (3.7, 4.0) | 3.9 (3.7, 4.1) | 4.1 (3.7, 4.3) | 3.8 (3.6, 4.5) |
| plasma hsCRP, mg/L | 4.2 (2.1, 7.9) | 4.2 (1.45, 5.5) | 9.5 (6.8, 12.6) | 19.5 ( 5.2, 21.3) |
| Plasma IL-6, pg/mL | 1.55 (0.91, 2.25) | 1.58 (1.22, 3.64) | 4.60 (4.35, 8.40) | 6.15 (1.83, 17.21) |
| Plasma TNF alpha, pg/mL | 1.67 (1.3, 3.14) | 2.1 (1.7, 3.6) | 21.6 (20.1, 23.1) | 20.7 (12.3, 66.6) |
| ***Lipids*** |  |  |  |  |
| High-density lipoprotein,mg/dl | 43 (35, 60) | 37 (31, 40) | 47 (45, 50) | 39 (35, 42) |
| Low-density lipoprotein,mg/dl | 83 (71, 123) | 78 (72,97) | 95 (87, 100) | 65 (39, 78) |
| Triglycerides, mg/dl | 115 (72, 144) | 147 (97, 184) | 104 (94, 130) | 203 (132, 267) |
| Total Cholesterol, mg/dl | 162 (129,190) | 143 (134, 179) | 160 (158,169) | 145 (126, 166) |

**TNF-**tumor necrosis factor alpha,**IL-6**: interleukin 6, **hsCRP:** high sensitive C reactive protein**.**

Statistical comparison done using chi-square for categorical variables and K-Wallis for continues variables**.**

Table 2: Change in mRNA expression (normalized) for cytokine and superoxide production

by LPS stimulated THP-1 macrophages

|  |  |  |  |
| --- | --- | --- | --- |
| **Biomarkers** | **Intervention** | **Placebo** | **Percent Change (p-value)\*** |
| **Study A (CKD stages 3 & 4)** | **Median (IQR)** | **Median (IQR)** |
| Baseline TNF- | 1.07 (0.92-1.17) | 1.10 (0.91-1.27) | -15% (p=0.05) |
| Post-treatment TNF- | 0.76 (0.68-0.99) | 0.85 (0.79-1.10) |
| Baseline IL-6 | 1.24 (0.96-1.42) | 1.24 (0.96-1.62) | -38% (p=0.004) |
| Post-treatment I-L6 | 0.93 (0.61-1.17) | 1.13 (1.05-1.59) |
| Baseline NLRP3 | 1.02  (0.77-1.10) | 0.94 (0.80-1.03) | -16% (p=0.01) |
| Post-treatment NLRP3 | 0.91 (0.57-0.99) | 0.93 (0.72-1.03) |
| Baseline superoxide | 490 (471-520) | 502 (469-513) | 17% (p<0.001) |
| Post-treatment superoxide | 513 (482-526) | 595 (574-610) |
| **Study B (maintenance hemodialysis )** | **Median (IQR)** | **Median (IQR)** | **Percent Change (p-value)** |
| Baseline TNF- | 0.83 (0.36-1.71) | 1.01 (0.34-2.67) | -64% (p=0.02) |
| Post-treatment TNF- | 0.66 (0.25-0.74) | 1.26 (0.69-1.60) |
| Baseline IL-6 | 1.26 (0.21-2.52) | 1.69 (0.15-3.69) | -56% (p=0.08) |
| Post-treatment IL-6 | 0.83 (0.17-0.95) | 1.63 (0.20-2.47) |
| Baseline NLRP3 | 0.93  (0.86-1.30) | 1.01 (0.82-1.41) | -25% (p=0.02) |
| Post-treatment NLRP3 | 0.77 (0.69-0.84) | 1.05 (0.91-1.10) |
| Baseline superoxide | 790 (766-813) | 780 (748-782) | 12% (p=0.004) |
| Post-treatment superoxide | 816 (788-840) | 921 (899-924) |

Cytokine response in LPS-stimulated THP-1 macrophages to HDL patients with CKD stages 3 & 4 treated with IL-1 trap (Study A) versus placebo and maintenance HD patients treated with IL-1ra (Study B) versus placebo.**(A)** IL-6 and (**B**) TNF- response before and after intervention in each trial. Values are expressed as median and interquartile range. mRNA expression measured by real-time PCR.

**TNF-**tumor necrosis factor alpha,**IL-6**: interleukin 6, **NLRP3**: Nod like receptor protein 3, **HD**: hemodialysis

The units for this biomarkers represent normalized mRNA in arbitrary units (AU).

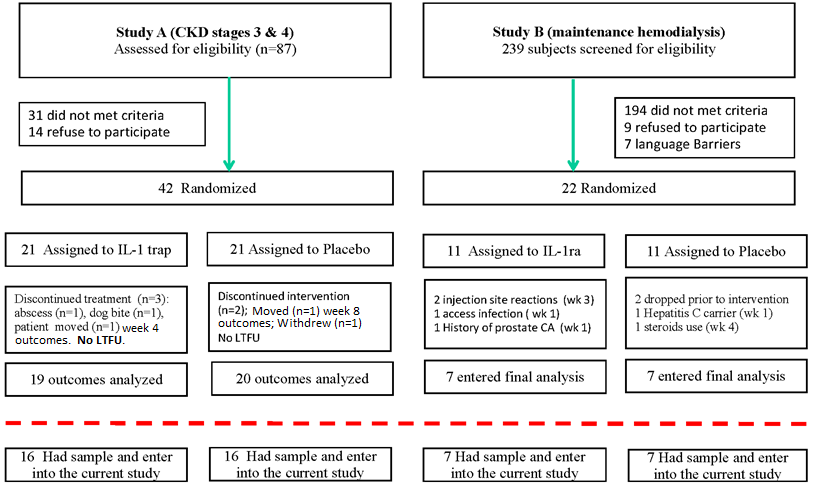
\*Statistical comparison of the intervention effect between groups drug versus placebo for each trial, was done using Analysis of covariance to estimate the percent change (ANCOVA). All variables were log transformed.

Table 3: Change in plasma lipid profile in the intervention group compared to the placebo group

|  |  |  |  |
| --- | --- | --- | --- |
| **Study A (CKD stages 3 & 4)** | Intervention | Placebo | p-value\* |
| Baseline total cholesterol, mg/dl | 162 (129,190) | 143 (134, 179) | P=0.22 |
| Post treatment total cholesterol, mg /dl | 168 (138, 201) | 144 (134, 174) |
| Baseline HDL, mg/dl | 43 (35, 60) | 37 (31, 40) | p=0.06 |
| Post treatment HDL, mg/dl | 47 (43, 50) | 35 (30,46) |
| Baseline LDL, mg/dl | 83 (71, 123) | 78 (72, 97) | p=0.35 |
| Post treatment LDL, mg/dl | 95 (74, 137) | 84 (71, 102) |
| Baseline triglycerides, mg/dl | 115 (72, 144) | 147 (97, 184) | p=0.91 |
| Post treatment triglycerides, mg/dl | 112 (83, 177) | 137 (102, 198) |
| **Study B (maintenance hemodialysis)** |  |  |  |
| Baseline total cholesterol, mg/dl | 160 (158,169) | 145 (126, 166) | P=0.51 |
| Post treatment total cholesterol, mg /dl | 170 (159, 185) | 152 (146,152) |
| Baseline HDL, mg/dl | 47 (45, 50) | 39 (35, 42) | p=0.02 |
| Post treatment HDL, mg/dl | 48 (42,56) | 35 (34, 35) |
| Baseline LDL, mg/dl | 95 (87, 100) | 65 (39, 78) | p=0.31 |
| Post treatment LDL, mg/dl | 92 (89, 101) | 61 (40, 74) |
| Baseline triglycerides, mg/dl | 104 (94, 130) | 203 (132, 267) | p=0.94 |
| Post treatment triglycerides, mg/dl | 113 (109, 178) | 213 (148, 353) |

\*Statistical Comparison of the intervention effect between groups drug versus placebo for each trial, was done using Analysis of covariance to estimate the percent change (ANCOVA). All variables were log transformed.

Figure 1 Consort Diagram



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Figure 2b

Figure 2a

**Figure 2**. Cytokine response in LPS-stimulated THP-1 macrophages to patients HDL before and after treatment with IL-1 trap versus placebo for Study A (CKD stages 3 & 4) and with IL-1ra versus placebo for Study B maintenance HD (MHD)). **(A)** IL-6 and (**B**) TNF- mRNA expression response before and after intervention in each trial measured by real-time PCR.

\*Statistical comparison of the intervention effect between groups drug versus placebo for each trial, was done using Analysis of covariance (ANCOVA) (p-values are derived from the ANCOVA).



**Figure 3.** NLRP3 mRNA expression in LPS-stimulated THP-1 macrophages exposed to patients HDL before and after treatment with, IL-1 trap versus placebo for Study A (CKD stages 3 & 4) and with IL-1ra versus placebo for Study B (maintenance hemodialysis). NLRP3 mRNA expression was measured by real-time PCR.

\*Statistical comparison of the intervention effect between groups, drug versus placebo for each trial, was done using Analysis of Covariance (ANCOVA) (p-values are derived from the ANCOVA).

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**Figure 4**. Cellular production of reactive oxygen species in LPS-stimulated THP-1 macrophages exposed to patients HDL before and after treatment with, IL-1 trap versus placebo for Study A (CKD stages 3 & 4) and with IL-1ra versus placebo for Study B (maintenance hemodialysis).

\*Statistical comparison of the intervention effect between groups, drug versus placebo for each trial, was done using Analysis of Covariance (ANCOVA) (p-values are derived from the ANCOVA).