Mild Renal Dysfunction and Metabolites Tied to Low HDL Cholesterol are Associated with Monocytosis and Atherosclerosis

Running title: Ganda et al.; Renal dysfunction, low HDL, monocytosis

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Abstract:

Background—The number of circulating blood monocytes impacts atherosclerotic lesion size and in mouse models, elevated levels of high density lipoprotein-cholesterol (HDL-C) suppress blood monocyte counts and atherosclerosis. We hypothesized that individuals with mild renal dysfunction at increased cardiovascular risk would have reduced HDL levels, high blood monocyte counts, and accelerated atherosclerosis.

Methods and Results—To test whether mild renal dysfunction is associated with increase in a leukocyte subpopulation rich in monocytes that has a known association with future coronary events, we divided individuals from the Malmö Diet and Cancer study (MDC) into baseline cystatin C quintiles (N=4757). Lower levels of renal function were accompanied by higher monocyte counts, and monocytes were independently associated with carotid bulb intima-media thickness cross-sectionally (p= 0.02). Cystatin C levels were positively and plasma HDL-C levels negatively associated with monocyte counts at baseline, following adjustment for traditional risk factors. Several amino acid metabolites tied to low HDL-C and insulin resistance measured in a subset of individuals (N= 752) using liquid chromatography-mass spectrometry were independently associated with a 22-34% increased risk of being in the top quartile of monocytes (p<0.05).

Conclusions—A low HDL-C, insulin resistance phenotype occurs in subjects with mild renal dysfunction and is associated with elevated monocytes and atherosclerosis. High blood monocytes may represent a previously unrecognized mechanism underlying the strong relationship between cystatin C and cardiovascular risk.

Key words: atherosclerosis, immunology, kidney, metabolomics, risk factors/global assessment
Patients with chronic kidney disease (CKD) have a markedly increased risk of atherosclerotic cardiovascular (CV) disease.\textsuperscript{1} The risk of CV events increases as the estimated glomerular filtration rate (eGFR) declines.\textsuperscript{2} While CKD may be associated with several well-known atherosclerosis risk factors, such as diabetes, hypertension, and elevated blood cholesterol, the atherosclerosis in patients with CKD is not fully explained by traditional risk factors.\textsuperscript{3} The recent SHARP trial (Study of Heart and Renal Protection)\textsuperscript{4} demonstrated a 17\% relative risk reduction in first major atherosclerotic event in 9270 CKD patients receiving simvastatin plus ezetimibe versus placebo, however event rates remained high in treated patients, and similar to two previous trials in hemodialysis patients, there was no significant reduction in mortality or non-fatal myocardial infarction from low density lipoprotein cholesterol (LDL-C) lowering.\textsuperscript{4-6} Therefore, LDL-C reduction deals with only a portion of CV risk in CKD, and additional causes of accelerated atherosclerosis must be explored in order to devise new treatments.

Multiple risk factors for coronary heart disease (CHD) have been identified in CKD beginning with mild impairments in renal function above an eGFR of 60 ml/min/1.73m\textsuperscript{2},\textsuperscript{3} however the significance and interrelationships among these risk factors are not well understood. Patients with mild renal dysfunction develop an unfavorable lipid profile characterized by rising triglyceride (TG) and declining high density lipoprotein cholesterol (HDL-C) concentrations,\textsuperscript{3, 7} each of which are independently associated with CHD.\textsuperscript{3} Atherogenic remnants of TG-rich lipoproteins accumulate as renal function deteriorates,\textsuperscript{8} and patients with CKD\textsuperscript{9} and End Stage Renal Disease (ESRD)\textsuperscript{10} are at increased risk for CV events\textsuperscript{9} and CV death\textsuperscript{10} from increasing atherosclerosis as shown by carotid intima-media thickness (IMT) measurements.\textsuperscript{9-10}

The number of circulating monocytes and their differentiation into lipid-laden macrophages in the arterial wall are fundamental events in plaque formation\textsuperscript{11-12} and in recent
years peripheral monocyte count has emerged as a strong and independent predictor of cross-sectional and future atherosclerosis in large population-based cohorts.\textsuperscript{13-14} In patients with ESRD on hemodialysis, total monocyte counts and certain monocyte subsets are increased cross-sectionally as compared to healthy controls,\textsuperscript{15} and small studies have shown that specific subsets are associated with CV events and mortality in ESRD\textsuperscript{16} and CKD.\textsuperscript{17} In addition, spikes in monocyte count to $>11\%$ of total leukocytes over time are associated with a composite endpoint of ESRD and death.\textsuperscript{18} However in pre-dialysis stages of CKD, these small studies have not shown increased total monocyte counts at baseline compared to subjects without CKD\textsuperscript{17-18} and to the best of our knowledge, a large-scale, detailed analysis of monocyte count as a marker of atherosclerosis in the setting of mild renal dysfunction has never been undertaken.

The ability of HDL to stimulate removal of cholesterol from macrophages, “cholesterol efflux”, is thought to be central to its anti-atherogenic mechanism.\textsuperscript{19} In mouse models, we recently discovered that the absence of ABCA1 and ABCG1, two ATP-binding cassette transporters that promote HDL-mediated cholesterol efflux,\textsuperscript{20} leads to proliferation of hematopoietic stem and progenitor cells (HSPCs), myeloid progenitor cells, and blood monocytes in association with accelerated atherosclerosis, and that transplantation of knockout bone marrow into apolipoprotein A1 (apoA1) transgenic mice with high HDL-C levels dramatically reverses this phenotype.\textsuperscript{20} We therefore hypothesized that individuals with mild renal dysfunction measured by elevated cystatin C (cysC) concentrations at increased CV risk\textsuperscript{21-22} might have reduced HDL-C levels, contributing to elevated monocyte counts. In addition, given recent studies showing that certain plasma metabolites predict characteristics of the metabolic syndrome\textsuperscript{23} and future diabetes,\textsuperscript{24} we explored whether several metabolic markers associated with low HDL-C\textsuperscript{23} would also be associated with the monocytosis of mild renal
dysfunction.

Methods

Study Population

All human study protocols were approved by the Institutional Review Board of Lund University (Sweden). All study participants provided written informed consent. The Malmö Diet and Cancer Study (MDC) is a prospective, population-based cohort that included 28,449 randomly selected men (born between 1923 and 1945) and women (born between 1923 and 1950) who underwent a baseline examination between 1991 and 1996. From this cohort, 6103 persons enrolled in 1991-1994 were randomly selected to participate in the MDC cardiovascular cohort (MDC-CC), which was designed to investigate the epidemiology of carotid artery disease. We excluded participants with prior myocardial infarction or stroke at baseline (n=143). Of the remaining participants, fasting plasma samples at baseline were available for 5400. Among these, complete data on conventional CV risk factors were available for 5220. In order to assess the cross-sectional clinical endpoints below, we divided 4757 MDC-CC individuals who had cysC measured at baseline into cysC quintiles.

Clinical Examination and Laboratory Assays

MDC participants underwent baseline history, examination, and laboratory assessment. Fasting EDTA plasma was frozen at −80°C immediately after collection. CysC, an endogenous substance freely filtered by the kidney, captures the association of mild renal dysfunction with CV risk better than creatinine-based GFR equations and is often preferred for use when assessing CV endpoints in these individuals. CysC, fasting levels of HDL-C and TG, the homeostasis model assessment of insulin resistance (HOMA-IR), and total and differential peripheral
leukocytes were measured as described in the Methods section of the online-only Data Supplement.

**Metabolite Profiling**

Metabolites were profiled from EDTA plasma collected at the baseline examination in 759 MDC-CC individuals using previously described methodology\(^{23-24}\) (see the Methods section in the online-only Data Supplement for details). These subjects were derived from a nested incident CV disease case-control study (N=506)\(^{27}\) with cases and controls matched by gender, age, and Framingham risk score,\(^{28}\) and a nested incident diabetes case-control study (N=326).\(^{24}\) From this pool of 832, subjects were excluded who had CV disease prior to the baseline examination, incomplete data on cysC, or had been in both studies above, leaving 759 individuals. There were 752 individuals with complete data on all covariates (Metabolite Cohort).

**Clinical Endpoints**

We primarily examined the surrogate CV endpoint top quartile of monocytes, measured at the time of the screening exam and defined in the online-only Data Supplement, which notably has been associated with future coronary events in an adjusted analysis of over 25,000 individuals from MDC.\(^{29}\) In MDC-CC, the top quartile of monocytes contained 0.70-1.80 million cells/mL, or a mean of 11% of total WBC. In addition, we examined a secondary endpoint at baseline: maximal carotid bulb IMT (IMT\(_{\text{max[Bulb]}}\)), measured in millimeters (further details provided in the online-only Data Supplement).

**Statistical Analysis**

All analyses were performed cross-sectionally at the time of the baseline visit. We divided 4757 MDC-CC subjects with baseline cysC into cysC quintiles and initially hypothesized 1) that
subjects in quintile 5 cysC would have the lowest HDL-C level and the highest monocyte count, and 2) that quintile 5 cysC and HDL-C would each independently be associated with the categorical primary outcome top quartile monocytes following multivariable adjustment for age, gender, quintiles 1-4 cysC, HOMA-IR, and current smoking, in a logistic regression model. Next, in 752 of these subjects, we explored the relationship of various candidate amino acid (AA) metabolites previously shown to have inverse associations with HDL-C\textsuperscript{23} to top quartile monocytes. Each metabolite was examined in a separate multivariable logistic regression model adjusted for age, gender, continuous (standardized) cysC, HDL-C, and HOMA-IR. All metabolite values were natural logarithmically transformed because of their non-normal distribution and then standardized (to mean=0, SD=1). Finally, utilizing a multivariable linear regression model adjusted for age, gender, quintiles 1-4 cysC, HOMA-IR, and current smoking, we hypothesized that quintile 5 cysC, HDL-C, and continuous monocytes would each independently be associated with the continuous secondary outcome \(\text{IMT}_{\text{maxBulb}}\) (log transformed due to skewed distribution). In all analyses, HOMA-IR was also log transformed due to skewed distribution.

All analyses were performed using SAS version 9.1.3 (SAS Institute, Cary, NC). Continuous variables are summarized as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to test for a difference in HDL-C, TG, monocyte, HOMA-IR, and AA means (respectively) across cysC quintiles. Results of logistic regression analyses are reported as odds ratio (OR) with 95% confidence interval (CI). Results of linear regression analyses are reported as standardized regression coefficients (\(\beta, \text{SE}\)). A two-tailed p value of <0.05 was considered statistically significant. Given the exploratory nature of the AA analyses, nominal significance testing (p<0.05) was used without correction of p values for multiple comparisons.
Results

Baseline characteristics of the MDC-CC human study sample and the Metabolite Cohort are shown in Table 1. Mean (± SD) age of subjects with complete data on conventional CV risk factors (N= 5220) was 58 ± 6 years, and 60% were women. Comparable age, gender distribution, and level of traditional risk factors was seen in the Metabolite Cohort (N=752).

MDC-CC Renal Demographics by Cystatin C Quintiles

We divided 4757 MDC-CC subjects with baseline plasma cysC into cysC quintiles (Table 2). Quintile 5 representing the highest cysC levels contained 992 individuals with cysC range 0.88-3.29 mg/L. Given the mean cysC of 0.99 ± 0.18 mg/L in this quintile, and the corresponding mean eGFR of 69 ± 15 ml/min/1.73m² (Table 2), we have designated quintile 5 cysC as “mild renal dysfunction”. Only 38 subjects had cysC ≥1.23 mg/L, which approximates an eGFR < 60 ml/min/1.73m², commonly accepted as CKD.²

Mild Renal Dysfunction is Linked to Low HDL-C Level and High Monocytes

In cross-sectional analysis of 4581-4757 MDC-CC individuals with available covariate data, lower levels of renal function marked by higher cysC concentrations were associated with lower fasting plasma HDL-C levels, and higher monocyte counts (Table 3, Figure 1A and C), p<0.001, respectively (ANOVA). The percent decrease in HDL-C and increase in monocytes with lower levels of renal function across cysC quintiles 1-5 were similar: 14% and 11%, respectively. Consistent with our primary hypothesis, individuals in quintile 5 cysC with mild renal dysfunction had the lowest plasma HDL-C level (49.4 ± 13.7 mg/dL) and the highest monocyte count (0.54 ± 0.19 million cells/mL). Since HDL-C levels are typically inversely correlated with plasma TG levels, we also explored the relationship of TG levels to cysC and indeed observed that individuals in quintile 5 cysC had the highest plasma TG level (139.7 ±
70.6 mg/dL), **Table 3** and **Figure 1B**, and the greatest insulin resistance (HOMA-IR score).

**Table 3.**

**Mild Renal Dysfunction is Linked to a High Risk Metabolic Profile**

In 759 MDC-CC individuals derived from a nested incident CV disease case-control study\(^27\) and a nested incident diabetes case-control study\(^24\) described above, we used an LC-MS based platform\(^23-24\) to ask whether branched-chain and aromatic AAs previously associated with insulin resistance and diabetes risk\(^23-24\) would be associated with worsening renal function. Higher cysC concentrations were associated with higher levels of the “Three AA score” (isoleucine+phenylalanine+tyrosine) and the “Five AA score” (isoleucine+phenylalanine+tyrosine+valine+leucine), which predict onset of future diabetes\(^24\) (**Table 3**), \(p=0.017\) and \(p=0.048\), respectively (ANOVA). Of note, in previously published linear regression analyses adjusted for age and sex, each individual AA above has a highly significant inverse relationship with plasma HDL-C, a significant positive relationship with plasma TGs, and various associations with other metabolic traits and insulin resistance phenotypes, highlighting the connection of the AAs we measured to metabolic risk.\(^23\) Individuals in quintile 5 cysC with mild renal dysfunction exhibited a high risk profile with elevated branched-chain and aromatic AAs, low HDL-C, high TGs, high HOMA-IR score, and high monocytes (**Table 3**).

**Mild Renal Dysfunction and Low HDL-C Level are Associated with Increased Risk of Being in the Top Quartile of Monocytes**

In MDC, the top quartile of monocytes, measured at the time of the baseline exam and defined above, is associated with future coronary events in an adjusted analysis of over 25,000 individuals.\(^29\) Therefore, we cross-sectionally examined the top quartile of monocytes as a surrogate CV endpoint in a multivariable logistic regression model containing age, gender, log-
transformed HOMA-IR, HDL-C, and quintiles 1-5 cysC (N= 4574, Table 4). Compared to quintile 1 cysC, quintile 5 cysC (mild renal dysfunction) was independently associated with 57% increased odds of being in the top quartile of monocytes (OR 1.570, 95% CI 1.215-2.029, p=0.001). Quintiles 2-4 cysC were not independently associated with the top quartile of monocytes. CysC remained associated with the top quartile of monocytes when entered into the multivariable model as a standardized continuous variable instead of divided into quintiles however the odds were attenuated (OR 1.155, 95% CI 1.072-1.246, p<0.001), confirming that individuals at greatest risk for increased monocytes were in quintile 5 cysC. HDL-C level was independently associated with 42% decreased odds of being in the top quartile of monocytes (OR 0.576, 95% CI 0.444-0.746, p<0.001). Female gender was also independently associated with 38% decreased odds (OR 0.624, 95% CI 0.528-0.737, p<0.001). Age and baseline HOMA-IR were not independently associated with the top quartile of monocytes (Table 4). Given the known strong association between cigarette smoking and monocyte count,\textsuperscript{31} we then further adjusted our logistic regression model for this variable. We discovered that both the increased odds of monocytosis associated with quintile 5 cysC (mild renal dysfunction), and the decreased odds of monocytosis associated with HDL-C level, were independent of current smoking and all other variables in the model (Table 4).

**Elevated Levels of Metabolites Tied to Low HDL-C are Associated with Increased Risk of Being in the Top Quartile of Monocytes**

In 752 MDC-CC individuals, we used an LC-MS based platform\textsuperscript{23-24} to assess whether certain AA metabolites tied to low plasma HDL-C\textsuperscript{23} were also independently associated with increased odds of being in the top quartile of monocytes at baseline (Table 5). Individual multivariable logistic regression models contained the log transformed and standardized candidate metabolite
of interest as well as age, gender, log-transformed HOMA-IR, cysC, and HDL-C. Tyrosine,
glutamate, carnitine, alanine, n-carbamoyl-β-alanine, allantoin, and dimethylglycine, each of
which has a known inverse association with HDL-C,23 were independently associated with 22-
34% increased odds of being in the top quartile of monocytes, p<0.05 for all. α-
glycerophosphocholine, which is positively associated with plasma TGs,23 was independently
associated with 23% increased odds of being in the top quartile of monocytes, p=0.02.
Interestingly, the “Three AA score” (isoleucine+ phenylalanine+tyrosine), the components of
which are inversely associated with plasma HDL-C and positively associated with plasma TGs,23
predicts onset of future diabetes24 and CV events,27 and increases as renal function declines
(Table 3). Each 1 standard deviation increase of the “Three AA score” was independently
associated with a 28% increased odds of being in the top quartile of monocytes (OR 1.281, 95%
CI 1.039-1.581, p=0.02). Glutamine, which is correlated with high plasma HDL-C, and
negatively associates with insulin resistance phenotypes,23 was nearly significantly associated
with 14% reduced odds of being in the top quartile of monocytes (OR 0.858, 95% CI 0.714-
1.030, p=0.1, Table 5).

Mild Renal Dysfunction, Low HDL-C Level, and Monocytes are Independently Associated
with Carotid Atherosclerosis

We examined the cross-sectional atherosclerosis endpoint IMT_{max}Bulb (N=3134), measured in
millimeters and log-transformed. A multivariable linear regression model evaluating this
atherosclerosis outcome contained age, gender, log-transformed HOMA-IR, HDL-C, quintiles 1-
5 cysC, and monocytes (Table 6). Age was independently and strongly associated with
IMT_{max}Bulb (β=0.24, p<0.001). In addition, monocytes were independently and significantly
associated with increased IMT_{max}Bulb, p=0.02, confirming the results of other large studies
linking monocytes to carotid IMT and atherosclerotic plaque formation.\textsuperscript{13-14} Mild renal dysfunction (quintile 5 cysC), compared to quintile 1 cysC, was also independently and significantly associated with increased IMT\textsubscript{maxBulb}, \textit{p}<0.01. Quintiles 2-4 cysC were not independently associated with IMT\textsubscript{maxBulb}. When cysC was entered into the multivariable model (containing monocytes and the other variables above) as a standardized continuous variable instead of divided into quintiles, it remained strongly and independently associated with IMT\textsubscript{maxBulb} (\textit{p}<0.01). Finally, low HDL-C level was independently associated with an increase (\textit{p}=0.02) and female gender with a decrease (\textit{p}<0.001) in IMT\textsubscript{maxBulb} (\textbf{Table 6}). Upon further adjustment of our linear regression model for current smoking, low HDL-C and mild renal dysfunction (quintile 5 cysC) remained significantly and independently associated with IMT\textsubscript{maxBulb}. The association of monocytes with atherosclerosis in this expanded model was overshadowed by the strong effect of smoking (\textbf{Table 6}); however, in a published analysis of over 25,000 MDC individuals, the top quartile of monocytes measured at the time of the baseline exam is associated with future coronary events independent of smoking.\textsuperscript{29}

\textbf{Discussion}

It is well established that individuals with early decrements in renal function, measured by cysC, are at increased risk for CV events and death\textsuperscript{21-22}; however, the mechanism underlying the strong relationship of cysC to CV risk has remained a matter of considerable debate.\textsuperscript{32-35} Due to the lack of independent association between cysC levels and carotid IMT in recent population-based studies,\textsuperscript{32-33} it has been suggested that in contrast to patients with CKD\textsuperscript{9} and ESRD,\textsuperscript{10} accelerated atherosclerosis may not be the primary mechanism explaining the independent relationship between cysC level and CV risk in individuals with early kidney disease.\textsuperscript{32-33} However, other
studies demonstrated that in individuals with an eGFR higher than 60 ml/min/1.73m², cysC is associated with early stage coronary atherosclerotic plaque morphology on multidetector computed tomography as well as coronary atherosclerosis extent by angiography, following adjustment for traditional risk factors. Because of the well-known association between cysC and CV events, and the fact that cysC concentrations perform better than creatinine-based equations in predicting GFR in individuals at higher levels of renal function, we hypothesized that mild renal dysfunction measured by cysC would be associated with elevated monocyte count at baseline, an important marker and likely mediator of atherosclerotic plaque formation that is increased in ESRD.

We now report in a cohort of over 4500 individuals that even mild levels of renal dysfunction are accompanied by higher levels of circulating monocytes, and compared to the first quintile cysC, the fifth quintile cysC in our study is strongly and independently associated with 44-57% increased odds of monocytosis at baseline, following adjustment for traditional risk factors. Consistent with our findings, a recent publication associated cysC to peripheral monocyte count in a small population sample (490 subjects), but did not relate monocytes to HDL-C levels or carotid IMT measurements. Our nearly 10 times larger study shows elevated monocyte counts in subjects with mild renal dysfunction and also demonstrates that low HDL-C and AA metabolites tied to low HDL-C are independently associated with the monocytosis of mild renal dysfunction. Moreover, we have shown a strong relationship of monocyte count with IMT at the carotid bifurcation, an area of low sheer stress prone to early plaque formation, suggesting a direct mechanism of accelerated atherogenesis in mild renal dysfunction. We propose that the largely unelucidated mechanisms underlying the relationship of cysC to CV risk involve increased circulating monocytes and low HDL-C level, leading to accelerated
atherosclerosis. Importantly, although ESRD patients on hemodialysis have elevated total monocyte counts compared to controls, small studies to date have not shown an increase in total monocyte counts at baseline in individuals with pre-dialysis stages of CKD. Our new findings raise the possibility that low HDL-C may be causally related to defective cholesterol efflux in HSPCs and myeloid cells of patients with mild renal dysfunction, promoting increased monocyte formation.

We made the novel discovery that individuals in the fifth quintile cysC exhibited a high risk metabolic profile with elevated branched-chain and aromatic AAs, and that isoleucine+phenylalanine+tyrosine not only forecasts diabetes and CV events, but is independently associated with a 28% increased odds of being in the top quartile of monocytes at baseline. Multiple other AA metabolites tied to low plasma HDL-C level and various other insulin resistance phenotypes (tyrosine, glutamate, carnitine, alanine, n-carbamoyl-β-alanine, allantoin, dimethylglycine) were also independently associated with increased odds of being in the top quartile of monocytes. Insulin resistance emerges with incipient renal disease and the combination of CKD plus the metabolic syndrome is associated with CV events. Our findings suggest that new sensitive markers of insulin resistance and the metabolic syndrome may represent key underlying factors contributing to increased monocytes in mild renal dysfunction.

One possible explanation for the relationship between AA metabolites and monocytosis relates to the mechanism by which nutritional factors contribute to insulin resistance. Exposure of cells to high physiologic concentrations of branched-chain AAs activate mammalian target of rapamycin (mTORC1) signaling pathways important for protein synthesis, and inhibit early steps in insulin action leading to decreased glucose use in skeletal muscle. mTORC1 is an evolutionarily conserved protein kinase that enhances cell growth and proliferation, and
suppresses autophagy, a degradative process in which intracellular contents are broken down in lysosomes to provide nutrients during periods of starvation.\textsuperscript{42} Interestingly, it was very recently discovered that autophagy is required for cholesterol efflux to HDL and apoA-1 from murine macrophage foam cells,\textsuperscript{43} suggesting that major pathways which suppress autophagy such as mTORC1 may be involved in defective cholesterol efflux and its downstream effects, including monocytosis. We therefore propose that AA-mediated activation of mTORC1\textsuperscript{41} may be involved in impaired HDL-mediated cholesterol efflux via suppression of autophagy\textsuperscript{43} and consistent with this hypothesis, we discovered that AA metabolites tied to low HDL-C\textsuperscript{23} were independently associated with increased odds of monocytosis.

Several limitations of our study warrant consideration. First, the automatic cell counter which we used did not differentiate among monocytes and basophils/eosinophils; however as previously published, the latter two leukocyte classes are rare compared to monocytes, and the same outcome that we have examined is associated with future coronary events in a large adjusted analysis.\textsuperscript{29} This finding plus the significant and independent association we found between this count and carotid IMT agree with literature linking monocytes to carotid atherosclerosis\textsuperscript{13-14} and CV events,\textsuperscript{17} and strengthen our results. HDL-C has previously been associated with monocyte count in studies one-third to one-fifth the size of ours, both in healthy individuals and in subjects with the metabolic syndrome.\textsuperscript{44-45} Our much larger study extends this primary observation to a new population, demonstrating that HDL-C plays an important role in the monocytosis of individuals with mild renal dysfunction measured by cysC. We additionally show that AA metabolites tied to low HDL-C and insulin resistance\textsuperscript{23} are associated with monocytosis, and demonstrate a strong relationship of monocyte count with carotid IMT, substantially implicating our findings in atherogenesis. The failure of HDL-elevating therapies
in recent clinical trials,\textsuperscript{46-47} as well as lack of a strong, direct relationship between HDL-elevating single nucleotide polymorphisms and CV disease in human genome-wide association studies\textsuperscript{48} has led to the suggestion that low HDL-C represents a risk marker only, possibly integrating effects of insulin resistance, hypertriglyceridemia, remnant accumulation, and other factors, without a direct causal relationship to atherogenesis.\textsuperscript{48} Whether low HDL-C levels directly contribute to monocytosis and atherosclerosis risk and/or represent a biomarker of metabolic risk cannot be discerned from our studies.

In conclusion, we provide important evidence that cysC is significantly and independently associated with monocytosis, and that the fifth quintile cysC and monocytes were each independently associated with carotid IMT, strongly suggesting that accelerated atherosclerosis is at least partly responsible for increased CV disease risk in mild renal dysfunction. Increased monocyte counts at baseline in individuals with mild renal dysfunction may arise from low HDL-C levels, possibly reflecting defective cholesterol efflux pathways, and monocytosis and HDL-C levels are related to a high risk AA signature forecasting diabetes\textsuperscript{24} and CV events.\textsuperscript{27} Elevated monocytes may provide a previously unrecognized and key mechanism for the strong link between cysC and CV risk.\textsuperscript{21-22}

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Conflict of Interest Disclosures: Drs. Gerszten and Wang are named as co-inventors on a patent application to the US Patent Office pertaining to metabolite predictors of diabetes mellitus. The other authors report no conflicts of interest.

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Table 1. MDC-CC: Baseline Characteristics

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<th>Metabolite Cohort† N=752</th>
<th>Complete Data on Conventional Cardiovascular Risk Factors* N=5220</th>
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<td>Current smoking, No. (%)</td>
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*Complete data on conventional cardiovascular risk factors at baseline was available in 5220 individuals. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; and HOMA-IR, homeostasis model assessment of insulin resistance.

† Baseline plasma metabolite profiling was performed in 752 individuals with complete covariate data.

Table 2. MDC-CC: Renal Demographics by Cystatin C Quintiles*

<table>
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<tr>
<th>CysC quintile</th>
<th>CysC range (mg/L)</th>
<th>CysC mean ± SD (mg/L)</th>
<th>eGFR (MDRD) mean ± SD (ml/min/1.73m²)</th>
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<td>0.68-0.73</td>
<td>0.71 ± 0.02</td>
<td>77.99 ± 14.02</td>
<td>927</td>
</tr>
<tr>
<td>3</td>
<td>0.74-0.79</td>
<td>0.77 ± 0.02</td>
<td>74.93 ± 14.49</td>
<td>932</td>
</tr>
<tr>
<td>4</td>
<td>0.80-0.87</td>
<td>0.83 ± 0.02</td>
<td>72.96 ± 13.68</td>
<td>904</td>
</tr>
<tr>
<td>5</td>
<td><strong>0.88-3.29</strong></td>
<td><strong>0.99 ± 0.18</strong></td>
<td><strong>68.84 ± 14.57</strong></td>
<td><strong>992</strong></td>
</tr>
</tbody>
</table>

* Baseline plasma cystatin C (cysC) was available in 4757 individuals who were divided into cysC quintiles. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) Study equation.
Table 3. MDC-CC: Metabolic Risk and Monocyte Count by Cystatin C quintiles

<table>
<thead>
<tr>
<th>CysC quintile (mg/L)</th>
<th>Three AA score† Mean ± SD (isoleucine+ phenylalanine+ tyrosine)</th>
<th>HOMA-IR* mean ± SD</th>
<th>HDL-C level* mean ± SD (mg/dL)</th>
<th>TG level* mean ± SD (mg/dL)</th>
<th>Monocytes* mean ± SD (million cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.19 ± 0.98</td>
<td>1.7 ± 1.9</td>
<td>57.6 ± 14.6</td>
<td>101.5 ± 57.8</td>
<td>0.48 ± 0.17</td>
</tr>
<tr>
<td>2</td>
<td>-0.07 ± 0.97</td>
<td>1.7 ± 1.9</td>
<td>54.7 ± 14.4</td>
<td>115.5 ± 64.6</td>
<td>0.50 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>-0.03 ± 0.97</td>
<td>2.0 ± 2.4</td>
<td>52.8 ± 13.8</td>
<td>122.8 ± 69.6</td>
<td>0.51 ± 0.18</td>
</tr>
<tr>
<td>4</td>
<td>0.15 ± 1.08</td>
<td>2.0 ± 2.2</td>
<td>51.1 ± 13.1</td>
<td>131.7 ± 85.5</td>
<td>0.51 ± 0.16</td>
</tr>
<tr>
<td>5</td>
<td>0.13 ± 0.96</td>
<td>2.6 ± 4.7</td>
<td>49.4 ± 13.7</td>
<td>139.7 ± 70.6</td>
<td>0.54 ± 0.19</td>
</tr>
</tbody>
</table>

| p-value | 0.017 | <0.001 | <0.001 | <0.001 | <0.001 |

* From 4757 subjects with baseline plasma cystatin C (cysC), the homeostasis model assessment of insulin resistance (HOMA-IR, N=4581), plasma high density lipoprotein-cholesterol (HDL-C, N=4662), triglycerides (TG, N=4709), and monocytes (N= 4574) were measured. Monocytes were derived from an automatic cell counter three part differential method which distinguished cells based on their size: lymphocytes, monocytes plus rare basophils/eosinophils, and neutrophils.

† Baseline plasma metabolite profiling was performed in 759 individuals with complete data on cysC. All amino acid (AA) metabolite variables are log transformed and standardized continuous variables.

Table 4. MDC-CC: Independent Associations with Top Quartile Monocytes*

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quintile 5 CysC</td>
<td>1.570</td>
<td>1.215-2.029</td>
<td>0.001</td>
<td>1.443</td>
<td>1.115-1.868</td>
<td>0.005</td>
</tr>
<tr>
<td>Quintile 4 CysC</td>
<td>1.120</td>
<td>0.861-1.458</td>
<td>0.397</td>
<td>1.069</td>
<td>0.821-1.393</td>
<td>0.620</td>
</tr>
<tr>
<td>Quintile 3 CysC</td>
<td>1.242</td>
<td>0.962-1.604</td>
<td>0.097</td>
<td>1.214</td>
<td>0.939-1.569</td>
<td>0.140</td>
</tr>
<tr>
<td>Quintile 2 CysC</td>
<td>1.097</td>
<td>0.846-1.422</td>
<td>0.486</td>
<td>1.074</td>
<td>0.827-1.394</td>
<td>0.592</td>
</tr>
<tr>
<td>Quintile 1 CysC</td>
<td>1.000</td>
<td>Referent</td>
<td>—</td>
<td>1.000</td>
<td>Referent</td>
<td>—</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.576</td>
<td>0.444-0.746</td>
<td>&lt;0.001</td>
<td>0.599</td>
<td>0.462-0.776</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (Female)</td>
<td>0.624</td>
<td>0.528-0.737</td>
<td>&lt;0.001</td>
<td>0.618</td>
<td>0.523-0.731</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>1.003</td>
<td>0.989-1.017</td>
<td>0.665</td>
<td>1.009</td>
<td>0.995-1.023</td>
<td>0.216</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.970</td>
<td>0.734-1.282</td>
<td>0.830</td>
<td>1.030</td>
<td>0.780-1.361</td>
<td>0.834</td>
</tr>
<tr>
<td>Current smoking</td>
<td>1.713</td>
<td>1.450-2.023</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All variables above were analyzed together in a multivariable logistic regression model (N= 4574) evaluating the outcome top quartile of monocytes. CysC indicates cystatin C. HDL-C indicates high density lipoprotein cholesterol. HOMA-IR indicates the homeostasis model assessment of insulin resistance (log-transformed).
Table 5: Independent Metabolite Associations with Top Quartile Monocytes*

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three AA score (isoleucine+phenylalanine+tyrosine)</td>
<td>1.281</td>
<td>1.039-1.581</td>
<td>0.021</td>
</tr>
<tr>
<td>Five AA score (isoleucine+phenylalanine+tyrosine+valine+leucine)</td>
<td>1.239</td>
<td>1.004-1.530</td>
<td>0.046</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.335</td>
<td>1.098-1.624</td>
<td>0.004</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.295</td>
<td>1.072-1.563</td>
<td>0.007</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.858</td>
<td>0.714-1.030</td>
<td>0.100</td>
</tr>
<tr>
<td>Carnitine</td>
<td>1.241</td>
<td>1.025-1.502</td>
<td>0.027</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.245</td>
<td>1.024-1.513</td>
<td>0.028</td>
</tr>
<tr>
<td>n-Carbamoyl-β-alanine</td>
<td>1.228</td>
<td>1.014-1.486</td>
<td>0.035</td>
</tr>
<tr>
<td>Allantoin</td>
<td>1.250</td>
<td>1.033-1.514</td>
<td>0.022</td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>1.220</td>
<td>1.013-1.470</td>
<td>0.036</td>
</tr>
<tr>
<td>a-Glycerophosphocholine</td>
<td>1.232</td>
<td>1.030-1.473</td>
<td>0.023</td>
</tr>
</tbody>
</table>

*Each amino acid (AA) variable above was analyzed in a separate multivariable logistic regression model evaluating the outcome top quartile of monocytes, adjusted for gender, age, log-transformed homeostasis model assessment of insulin resistance (HOMA-IR), high density lipoprotein cholesterol, and continuous standardized cystatin C (N=752). All metabolite variables are log transformed and standardized continuous variables. 10 other individual metabolites were analyzed which did not reach statistical significance: proline, adenosine, choline, serotonin, taurine, trimethylamine-N-oxide, phenylalanine, isoleucine, leucine, and valine.

Table 6. MDC-CC: Independent Associations with Carotid Bulb IMT*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standardized β Coefficient (SE)</th>
<th>p-value</th>
<th>Standardized β Coefficient (SE)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.243 (0.001)</td>
<td>&lt;0.001</td>
<td>0.260 (0.001)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (Female)</td>
<td>-0.082 (0.006)</td>
<td>&lt;0.001</td>
<td>-0.085 (0.006)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.039 (0.016)</td>
<td>0.024</td>
<td>0.024 (0.016)</td>
<td>0.173</td>
</tr>
<tr>
<td>HDL-C</td>
<td>-0.047 (0.009)</td>
<td>0.019</td>
<td>-0.040 (0.009)</td>
<td>0.044</td>
</tr>
<tr>
<td>Quintile 5 CysC</td>
<td>0.072 (0.009)</td>
<td>0.002</td>
<td>0.055 (0.009)</td>
<td>0.018</td>
</tr>
<tr>
<td>Quintile 4 CysC</td>
<td>0.017 (0.009)</td>
<td>0.455</td>
<td>0.007 (0.009)</td>
<td>0.761</td>
</tr>
<tr>
<td>Quintile 3 CysC</td>
<td>-0.002 (0.009)</td>
<td>0.915</td>
<td>-0.008 (0.009)</td>
<td>0.722</td>
</tr>
<tr>
<td>Quintile 2 CysC</td>
<td>0.008 (0.009)</td>
<td>0.725</td>
<td>0.003 (0.009)</td>
<td>0.894</td>
</tr>
<tr>
<td>Quintile 1 CysC</td>
<td>Referent</td>
<td>—</td>
<td>Referent</td>
<td>—</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.002 (0.011)</td>
<td>0.895</td>
<td>0.012 (0.010)</td>
<td>0.508</td>
</tr>
<tr>
<td>Current smoking</td>
<td><strong>0.122 (0.006)</strong></td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All variables above were analyzed together in a multivariable linear regression model (N= 3134 for the outcome log-transformed maximal carotid bulb IMT). IMT indicates intima-media thickness; HDL-C, high density lipoprotein cholesterol; CysC, cystatin C; and HOMA-IR, the homeostasis model assessment of insulin resistance (log-transformed).
Figure Legend:

**Figure 1.** Mild renal dysfunction is linked to low HDL-C level, high TG level, and high monocytes. In cross-sectional analysis of 4662-4757 MDC-CC individuals with available covariate data, lower levels of renal function marked by higher cystatin C concentrations were associated with lower fasting plasma high density lipoprotein (HDL) levels (A), higher fasting plasma triglyceride levels (B), and higher monocyte counts (C), p<0.001 for each (ANOVA). Results are shown as the mean of each variable by cystatin C quintile, with error bars representing the 95% confidence interval (CI). Monocytes were derived from an automatic cell counter three part differential method which distinguished cells based on their size: lymphocytes, monocytes plus rare basophils/eosinophils, and neutrophils.
Mild Renal Dysfunction and Metabolites Tied to Low HDL Cholesterol are Associated with Monocytosis and Atherosclerosis
Anjali Ganda, Martin Magnusson, Laurent Yvan-Charvet, Bo Hedblad, Gunnar Engström, Ding Ai, Thomas J. Wang, Robert E. Gerszten, Olle Melander and Alan R. Tall

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SUPPLEMENTAL MATERIAL

Supplementary Methods

Clinical Examination and Laboratory Assays.
MDC participants underwent baseline history, examination, and laboratory assessment.
Hypertension was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure (≥ 90 mmHg, or use of antihypertensive therapy. Glucose was measured in venous whole blood. Diabetes was defined as fasting blood glucose ≥ 6.1 mmol/L (corresponding to fasting plasma glucose of ≥ 7.0 mmol/L or 126 mg/dL), a self-reported physician diagnosis of diabetes, or use of anti-diabetic medication. Cigarette smoking was elicited by a self-administered questionnaire, with current cigarette smoking defined as any use within the past year.

Fasting EDTA plasma was frozen at −80°C immediately after collection. CysC was analyzed using a particle-enhanced immunonephelometric assay (N Latex Cystatin C; Dade Behring, Deerfield, Illinois).1-2 Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) Study equation.3 Fasting levels of HDL-C and TG were available in 4662 and 4709 of the 4757 individuals with baseline cysC and were measured according to standard procedures at the University Hospital Malmö. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as [fasting insulin (μU/ml) x fasting glucose (mmol/l)] / 22.5 as previously described4 and was available in 4581 individuals.

Total and differential peripheral leukocytes were counted using a SYSMEX K1000 automatic cell counter (Sysmex Europe, Norderstedt, Germany). The analyses were performed at the
time of the screening examination, at the University Hospital Malmö, using fresh heparinized blood. The leukocyte histogram was discriminated into small, middle, and large sized leukocytes by a three-part differential method, providing the following information in 1 uL of whole blood: absolute count of lymphocytes (small cells), absolute count of monocytes plus basophils/eosinophils (middle sized cells), and absolute count of neutrophils (large cells). The baseline count of middle sized cells consists mainly of monocytes, and in line with smaller studies linking monocytes to atherosclerosis and CV events, it has recently been shown to predict future coronary events in a large adjusted analysis of over 25,000 individuals. We shall refer to this count as monocytes, given the relative scarcity of basophils/eosinophils compared to monocytes in this count. Monocytes were available in all 4757 MDC-CC individuals with baseline cysC.

**Metabolite Profiling.** Plasma samples were stored at -80°C and profiled using liquid chromatography-tandem mass spectrometry (LC-MS) as described previously. LC-MS was performed using a 4000 QTRAP triple quadrupole mass spectrometer (AB SCIEX, Foster City, CA), coupled to a multiplexed LC system including two 1200 Series pumps (Agilent Technologies, Santa Clara, CA) and an HTS PAL autosampler (Leap Technologies, Carrboro, NC) with 2 injection ports and a column selection valve. The two pumps were similarly configured for hydrophobic interaction chromatography (HILIC) using 150 x 2.1 mm Atlantis HILIC columns (Waters; Milford, MA). MultiQuant software v1.1 (AB SCIEX, Foster City, CA) was used for automated peak integration and metabolite peaks also were manually reviewed for quality of integration. Formic acid, ammonium acetate, LC-MS grade solvents, and valine-d8 were obtained from Sigma-Aldrich (St. Louis, MO), with the remainder of isotopically-labeled analytical standards obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Internal standard peak areas were monitored for quality control and individual samples with
peak areas differing from the group mean by more than 2 standard deviations were re-analyzed. A complete list of metabolites analyzed is given in the sub-text of Table 5. Metabolites were selected based on the following criteria: 1) known structural identity; 2) distribution across multiple biochemical pathways; 3) reliable measurement using LC-MS in a high throughput fashion; and, 4) low rate of missingness on our platform (<1%). Using sample preparation and MS replicates of human samples, we have previously documented coefficients of variation (CV) for the metabolites in the platform: 54% of metabolites have CV ≤ 10% and 74% have CV ≤ 20%.¹⁹

**Clinical Endpoints**

We primarily examined the top quartile of monocytes, measured at the time of the screening exam and defined above. We examined a secondary endpoint at baseline: maximal carotid bulb IMT (IMT<sub>maxBulb</sub>). Using B-mode ultrasound, the right carotid bifurcation was scanned within a pre-defined “window” comprising 3 cm of the distal common carotid artery, the bifurcation, and 1 cm of the internal and external carotid artery, respectively. The maximum thickness of the intima–media (max IMT bifurcation) in the far wall of the carotid bifurcation was measured off-line according to the leading edge principle, using a specially designed computer-assisted image analyzing system as previously described.¹¹ IMT<sub>maxBulb</sub> (measured in millimeters) was available in 3134 MDC-CC participants with available covariate data.

**References for Supplementary Methods**


6. Chapman CM, Beilby JP, McQuillan BM, Thompson PL, Hung J. Monocyte count, but not c-reactive protein or interleukin-6, is an independent risk marker for subclinical carotid atherosclerosis. Stroke. 2004;35:1619-1624


