Differential Gene Transcription in Monocytes from Chronic Kidney Disease and Healthy Patients

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Abstract

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Chronic kidney disease (CKD) is a major cause of morbidity and mortality in the US. The prevalence of CKD currently is about 14%, or about 26 million people, and the number of deaths from CKD has doubled over the past 20 years. A significant number of CKD patients demonstrate accelerated cardiovascular disease (CVD). The importance of monocytes in development of CVD is well established. The purpose of the current pilot study was to compare gene transcription in monocytes from early stage CKD subjects with matched Healthy controls, focusing on genes linked to cardiovascular events. Our data support differential gene transcription in monocytes from CKD subjects, which translate to increased survivability, due in part to their reduced apoptotic signaling and increased membrane remodeling. CKD subject monocytes exhibited increased inflammatory signaling and oxidative stress compared with Healthy controls.

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List of Major Abbreviations

Abbreviation	Name
ACACA	Acetyl-CoA carboxylase
CACS	Coronary artery calcium score
ChREBP	Carbohydrate responsive element binding protein
CKD	Chronic kidney disease
CVD	Cardiovascular disease
ECM	Extracellular matrix
ESRD	End-stage renal disease
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FGF23	Fibroblast growth factor 23
GGPP	Geranylgeranyldiphosphate
HIF	Hypoxia inducible factor
IMT	Intima-media thickness
INSIG	Insulin induced gene
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LVH	Left ventricular hypertrophy
LXR	Liver X receptor
MAC-1	Macrophage adhesion ligand-1
MCP-1	Monocyte chemoattractant protein-1
MMP	Metalloproteinases
NKF	National kidney foundation
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF2	Nuclear Regulatory Factor-2
OPG	Osteoprotegerin
PPAR	Peroxisome proliferator- activated receptor
PWV	Pulse wave velocity
ROS	Reactive oxygen species
RANK	Receptor Activator of Nuclear Factor-Kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligand
RXR	Retinoid X receptor
SREBP	Sterol regulatory element-binding protein
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

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I. INTRODUCTION

A longitudinal observational multi-site cohort study, the Seattle Kidney Study, is ongoing. The purpose of this study is to understand the natural history of morbidity and mortality associated with kidney disease prior to initiation of dialysis, with the goal of reducing the high rate of adverse outcomes among patients on the chronic kidney disease (CKD) continuum. The primary objective of the research is to estimate the risks of adverse outcomes among patients with CKD and to identify conventional and non-conventional factors that contribute to overall risk assessment among patients with CKD. Of special interest are mortality and morbidity due to cardiovascular disease, infection, and fracture.

Due to the increasing prevalence of CKD and the strong link noted between CKD and cardiovascular disease (CVD), a pilot study was undertaken to investigate the genetic basis for the interrelatedness of risk factors and outcomes. Monocytes play a pivotal role in the progression of CKD and CVD, thus the pilot study evaluated transcription data from circulating monocytes from 3 subjects in the Seattle Kidney Study, compared with 3 matched Healthy controls, to better understand the interconnectedness of CKD and CVD. Subjects from early stage CKD were recruited in order to increase the potential of identifying CVD therapeutic targets for CKD subjects

II. BACKGROUND

1. CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) is a major cause of morbidity and mortality in the US. The prevalence of CKD currently is about 14%, or about 26 million people, and the number of deaths from CKD has doubled over the past 20 years.[1,2] In the United States, the prevalence of CKD increases with age. Using NHANES data, Hoerger et al. estimated the prevalence of CKD as about 4% of individuals between 30 and 49 years of age. Between 50 and 64 years of age, the prevalence increases to about 10%, and for people over 65 years of age, the prevalence is approximately 40%.[1]

The severity of CKD is defined by abnormalities of kidney structure or function that are present for more than 3 months. According to clinical practice guidelines published by the National Kidney Foundation (NKF) US-focused commentary on Kidney Disease Improving Global Outcomes (KDIGO), classification of patients with chronic renal insufficiency is based

on categories defined by glomerular filtration rate (GFR) and albuminuria. The disease is divided into 6 GFR categories, with severity for each category based on albumin excretion rate (A1: <30 mg/24 hours; A2: 30-300 mg/24 hours; A3: >300 mg/24 hours), as shown in Table 1. These clinical values provide a prognosis of CKD. For example, patients with a G1 category with A1 would be at normal to mildly increased risk, patients with A2 would be at moderately increased risk, and patients with A3 would be at severely increased risk of worsening CKD.[3]

Table 1: GFR categories in CKD

GFR category	GFR (ml/min/1.73 m^2)	Description
G1	≥ 90	Normal or high
G2	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	<15	Kidney failure

In the absence of evidence of kidney damage, neither GFR category G1 or G2 fulfill the criteria for CKD.[3]

The primary causes of CKD globally are diabetes and hypertension; these diseases account for 71% of CKD cases in the United States.[4] In contrast, glomerulonephritis accounts for nearly 25% of CKD cases in Japan, 33% of cases in China, and 41% of cases in Nigeria, but only 7% of CKD cases in the United States.[4]

CKD requires substantial US health care resources and is accompanied by high medical costs, which escalate in older adults.[5] For example, end-stage renal disease (ESRD), synonymous with a GFR of 5-10 ml/min/1.73 m^2 and the need for dialysis, cost Medicare $32.9 billion in 2010,[5] and earlier stages of CKD cost Medicare an estimated $48 billion in 2010.[1,6]

2. Interaction of CKD and CVD

A significant number of CKD patients demonstrate accelerated cardiovascular disease (CVD). Although CVD is the leading cause of death in the United States, meta-analyses suggest that there is an increased mortality of 1.4–3.7 times from CVD among patients with CKD.[2,7] In ESRD patients, who are dialysis-dependent, the risk of cardiovascular mortality is 10-fold to 20-fold higher than in patients without CKD.[8,9]

Kidney disease and CVD are interrelated, in that disease in one system generates dysfunction in the other, with accelerating disease progression in both.[10] The interconnectedness of CKD and CVD is largely due to clustering of risk factors for CKD, including anemia, volume overload, abnormal mineral metabolism, proteinuria, oxidative stress, and inflammation, and risk factors for CVD, including age, hypertension, diabetes, and dyslipidemia. The hazard ratio for cardiovascular events increases progressively from 1.0 at GFR >60 to 3.4 at a GFR <15.[10] According to Keith et al., a patient diagnosed with stage 3 CKD is at a higher risk of dying from CVD than of beginning renal replacement therapy.[11] Conversely, reversal of renal function by renal transplantation results in significant improvement in cardiac function.[12]

There are several theories for the increased risk of CVD in CKD, including increased oxidative stress, inflammation, platelet dysfunction, accelerated atherosclerosis, and reduced responsiveness to anti-platelet agents.[13-17] The most significant cardiac abnormality in CKD and ESRD patients is related to left ventricular (LV) structure and function.[18] About 75% of ESRD patients starting dialysis suffer from left ventricle hypertrophy (LVH), and another 15% have LV systolic dysfunction.[19]

Heart disease primarily presents in two ways in patients with CKD: atherosclerotic vascular disease, especially coronary artery disease (CAD), and LVH. Atherosclerosis is progressive with reduced GFR.[20,21] A large portion of dialysis patients with coronary disease are asymptomatic. deFilippi et al. reported 44% of asymptomatic dialysis patients had coronary disease.[22] Similarly, Ohtake et al. reported 53% of asymptomatic dialysis patients had coronary disease, as defined by >50% stenosis in the coronary artery.[23] Heart disease also presents as LVH. Herzog et al. reported 80% of CKD patients exhibit LVH at the time they begin dialysis.[24,25]

Several studies argue that cardiomyopathy is the significant CVD contributor in CKD. Glassock et al. suggest that factors other than atherosclerotic coronary artery disease (and lipid-rich plaques) are major contributors to mortality in ESRD. They state that ischemic cardiac disease, especially coronary artery atherosclerosis, is less important in the mortal and morbid cardiovascular consequences of CKD and ESRD. Interventions that address atherosclerotic complications of CKD and ESRD may be more effective if they account for LVH and cardiac fibrosis in reducing CV mortality in these patients.[26] Ritz and Bommer argue that the most frequent cause of cardiac death in dialysis patients is not from CHD (in contrast with the general population, where 80% of sudden deaths are from CAD), but rather from cardiomyopathy, which

occurs very early in the course of impaired renal function. Hypertrophy is characterized by interstitial fibrosis and capillary deficit.[27] According to Gross and Ritz, the primary role of arteriosclerosis in LVH is its contribution to central artery stiffness. A recent interventional study demonstrated the importance of arterial stiffness as a determinant of LVH by suggesting that alteration of aortic stiffness was the principal factor responsible for longitudinal changes in LV mass.[28]

3. CARDIOMYOPATHY

Cardiomyopathy is a result of many factors in CKD. For example, renal dysfunction causes retention of sodium and water, and thereby hypertension, resulting in increased systolic pressure. The LV must work against the increased pressure when it contracts. Renal dysfunction also results in reduced catecholamine clearance to activate sympathetic nerves.[29] In addition, protein-bound uremic toxins (PBUT) are increased with decreasing kidney function. PBUT, like indoxyl sulfate and p-cresyl sulfate, cause cardiovascular and renal injury by inducing oxidative stress as well as other pathologies.[30,31]

Myocardial hypertrophy and fibrosis may lead to a reduction in the capillary density and coronary reserve,[32,33] which result in ischemia[34] and increase the risk of ventricular arrhythmias and sudden cardiac death.[35] Ischemia is known to promote cardiomyocyte apoptosis and accumulation of extracellular matrix and collagen, thereby causing fibrosis, leading to LV stiffness, increased LV filling pressure, impaired diastolic filling, and diastolic dysfunction.[36]

Erythropoietin, produced in the kidneys, also appears to have a role in development of CVD. Erythropoietin has anti-apoptotic, anti-oxidant, and anti-inflammatory effects and helps protect cardiac, renal, and vascular cells. During heart failure, insensitivity to erythropoietin causes further inflammation.[37] In addition, erythropoietin deficiency exacerbates cardiorenal dysfunction by not only inducing ischemia but also reducing cell protective actions.[38-40]

Hyperphosphatemia, a prevalent condition in CKD, is associated with increased LV mass[41] and diastolic dysfunction,[42] contributing to the development of LVH and cardiomyopathy in CKD patients.[32] Angiotensin II accumulates in the heart and promotes myocyte hypertrophy, interstitial fibrosis and microvascular disease.[43] Elevated serum aldosterone, as a result of renin-angiotensin system activation or other means, can induce myocardial fibrosis, possibly by transforming growth factor b (TGFb) release.[35] In the CKD patients, heart failure (HF) is the

most common cardiac presentation, while LVH is predictive of CV mortality.[10] The primary cardiovascular pathologies in CKD patients, cardiac interstitial fibrosis and non-obstructive vascular diseases, are independent of hypertension [32,33] but still contribute to the incidence of heart failure in the absence of atherosclerosis in CKD patients.[10]

Toba et al. argues that CKD cardiovascular effects are similar to age-related changes. For example, age-related artery changes include endothelial dysfunction, wall thickening, and diameter narrowing.[31] The presence of endothelial dysfunction is an independent risk factor for progression of heart failure as well as the development of adverse outcomes after heart failure.[44,45] Arterial elasticity is needed to support expansion during systole and delivery of blood to the coronary arteries during diastole. Arterial stiffness of the coronary artery impairs coronary blood flow because the coronary arteries are perfused during diastole.[46] Additionally, increased stiffness of the larger arteries increases systolic pressure.[47]

4. ARTERIAL STIFFNESS

Arterial stiffness describes the reduced capability of an artery to expand and contract in response to pressure changes primarily caused by heart beats.[48] In the arterial system, the result of decreased elasticity is an increase in the propagation velocity of the pressure pulse along the arterial vessels, called the pulse wave velocity (PWV). Increased PWV results in pressure waves reflecting back to the aorta too soon and causing the LV to contract more strongly in order to overcome them, thereby momentarily increasing systolic pressure or systolic afterload.[48]

Arterial stiffness increases with age, either as a linear relationship between arterial stiffness and age,[49] or with accelerated stiffening between 50 and 60 years of age.[50] In contrast, stiffness of peripheral arteries increases less[49] or not at all with increasing age.[51]

Increased arterial stiffness of central arteries is found in both early[52] and advanced stages of CKD.[53] Arterial stiffness correlates with LVH[54] and its progression over time.[55] Prospective studies in ESRD patients have demonstrated that aortic stiffness is an independent predictor of all-cause and cardiovascular mortality.[56]

Stiffening is accomplished through remodeling of these arteries by hypertrophy of both the intima and the media, breaks and disordered bedding of elastic fibers, as well as calcification. Similar changes in arteriosclerosis are seen with aging and are characterized by diffuse over-

stretching of arteries, stiffening of the central arteries, and increased calcium deposits in the media layer.[28]

Age-related structural change in the aorta include both the media and intima. Changes in the media are fracturing of elastin and increasing collagen and calcium deposits. In the intima, the primary changes are an increase in intima-media thickness (IMT) and the prevalence of atherosclerotic plaques. Notable is that age-related increases in PWV have been found in populations with low prevalence of atherosclerosis. These results suggest that medial degeneration is an important cause of arterial stiffening. [48]

Arterial stiffness of ageing arterial media is associated with increased expression of matrix metalloproteinases (MMP), which are members of the zinc-dependent endopeptidase family. One of their functions is degradation of vascular elastin and collagen fibers. Although there are numerous types of MMPs, MMP2 and MMP9 are the most studied in relation to arterial stiffness.[48]

5. ATHEROSCLEROSIS

Although atherosclerosis is the fundamental cause of heart attack and stroke,[57] its contribution to the pathogenesis of CVD in CKD is less certain.[26] People with CKD respond differently to treatment of atherosclerosis than those without CKD. For example, Kim et al. reported that low-dose aspirin was significantly associated with increased risk of atherosclerotic event and a doubling of creatinine levels.[17] Although some report statins are not effective in CKD, several investigators report similar effectiveness.[58]

To briefly summarize atherosclerosis, elevated levels of LDL cholesterol and apolipoprotein B (apoB) 100, the main structural protein of LDL, are directly associated with risk for atherosclerotic cardiovascular events, such as stroke and heart attack.[59] When apoB lipoproteins infiltrate artery walls, they initiate an inflammatory response causing endothelial dysfunction, monocyte infiltration, macrophage activation, oxidative stress, and the development of foam cells initiated through the ingestion of oxidized lipoproteins by macrophages.[60] To counter the developing inflammatory accumulation of cholesterol, HDL, apoA-I, and endogenous apoE attenuate inflammation and oxidative stress, promote cholesterol efflux, and reduce lesion formation.[61,62]

Macrophages produce inflammatory chemo-attractants, encouraging infiltration and proliferation of smooth muscle cells, which produce the extracellular matrix that provide a stable barrier between plaque prothrombotic factors and platelets.[63,64] If inflammation is not resolved, there is increased macrophage apoptosis, defective efferocytosis of apoptotic cells resulting in necrotic cell death and leading to increased smooth muscle cell death, decreased extracellular matrix production, and collagen degradation by macrophage proteases.[65] Finally, the barrier ruptures, resulting in thrombus formation.[66,67]

The pathogenesis of atherosclerosis is primarily in the intima and is characterized by lipid accumulation, inflammatory cells, vascular smooth muscle cell (VSMC) migration, foam cell development, connective tissue fibers and calcium deposits. The resulting advanced plaque includes a lipid core, fibrous connective tissue deposits and calcification.[68] In the presence of atherosclerosis, stiffness of large arteries is increased.[69]

Stary et al.[70] suggest that increased IMT is indicative of early stages of atherosclerosis, because increased IMT is located in areas susceptible to atherosclerosis; because it predicts plaque development;[69] and because both IMT and presence of plaque are more prevalent in cardiovascular disease.[71] IMT is characterized by an accumulation of VSMC, elastin and proteoglycans, but not lipid deposits.[72]

Studies examining a correlation between the degree of IMT thickening or the presence of plaque and arterial stiffness are conflicting. Paini et al[73] reported both increases and decreases in stiffness for arterial regions containing plaque in humans, while Farrar et al.[74] showed initial decreases followed by increases in PWV for cynomolgus monkeys fed atherogenic diets. Cecelja and Chowienczyk conclude that these results suggest atherosclerotic change within the arterial wall is not necessarily associated with arterial stiffening.[48]

6. CALCIFICATION

Arterial calcification is the deposition of calcium-phosphate crystals in the media or intima of the arterial wall. The calcium content in arterial walls increases with age and accelerates after approximately 70 years of age.[75] Calcium deposits in the arterial wall are increased in end-stage renal disease and diabetes mellitus. Atherosclerotic plaques show an intimal focus of calcification. Medial calcification (also known as Monckeberg's sclerosis) is more diffuse, generally is associated with elastic fibers and commonly affects the aorta and femoral arteries.

London et al. reported higher PWV in individuals with medial and intimal calcification in end-stage renal disease.[76]

PWV is significantly associated with aortic calcification. [48] However, animal models consistently report a lack of association between PWV and early stages of atherosclerosis. In a study using female twins, calcified plaques, but not lipid-rich non-calcified plaques, correlate with increased stiffness. suggesting that the relation of PWV to vascular calcification may be independent of total plaque burden.[77,78] Marfan's syndrome is genetic disorder caused by a mutation of the FBN1 gene that encodes fibrillin-1. Fibrillin-1 regulates assembly of elastin fibers and is associated with increased arterial stiffness.[79]

The impact of calcification in the coronary artery on survival as measured by a coronary artery calcium score (CACS, measured in Agatston units) was analyzed in Nutritional and Inflammatory Evaluation of Dialysis Patients study.[80] This study was prospective and included hemodialysis patients. Groups were divided based on three levels of CACS score. No differences in serum calcium, phosphate, cytokine profile or BMI were observed between the groups. The hazard ratio of death in the CASC groups increased as the CASC score increased (1-100: HR 2.9; 101-400: HR 8.5; 400+: HR 13.3) compared to the reference group where CACS was zero. This study reported that CACS measured for each coronary artery (individual CACS) was predictive for all-cause mortality.[80]

A recent review noted that several studies have reported the inverse relationship between vascular calcification, vascular stiffness and bone mineral density in the general population.[81] A similar relationship has been reported in patients with CKD.[82] Low bone turnover is associated with advanced vascular calcification in CKD patients.[83] Osteoprotegerin (OPG), which is a receptor activator of Nuclear Factor-Kappa B (NF-KB) ligand (OPG/RANKL) via Receptor Activator of NF-KB (RANK) and is important in regulation of bone resorption, was also postulated to be involved in soft tissue calcification in uremia. RANK is a member of the tumor necrosis factor (TNF)-α superfamily, thus its activation promotes inflammatory gene and cytokine expression through NF-KB and JUN N-Terminal Kinase (JNK) activation.[84-86] TNF-α levels also correlate with CKD, coronary artery disease, and arterial calcification.[87] A recent study reported that serum OPG was higher for hemodialysis (HD) patients than for stage 4 CKD or controls, while RANKL levels decreased with increasing severity of CKD, resulting in a significantly higher OPG/RANKL ratio in hemodialysis versus stage 4 CKD or controls. Both

the OPG and OPG/RANKL ratio correlated with coronary artery calcification at baseline and after 1 year observation. Multivariate analysis confirmed an independent relationship between the progression of coronary artery calcification and serum OPG.[88]

7. PHOSPHATE CALCIUM

People with CKD display abnormal mineral and bone metabolism from the early stages of disease, which presents a strong cardiovascular risk for CKD patients. One of the primary characteristics is hyperphosphatemia, while levels of calcium and parathyroid hormone (PTH) are variable. Phosphate contributes to the development of artery mineralization both as a substrate, which can be deposited in vessels within the medial or intimal layers and as a mediator for activating gene transcription in VSMC and pericytes, transforming them into osteoblast-like cells. Mineral deposition in vessel walls is regulated similar to bone formation. For example, macrophages resembling osteoclasts can be found in areas of vascular mineralization. Unlike bone formation, however, ossification is not balanced by resorption in vessel walls.[89,90] High serum phosphate is associated with increased risk of CV endpoints in CKD and the general population as well,[91-93] while low phosphate is associated with less calcification in artery walls.[94]

Fibroblast growth factor 23 (FGF23), a class of proteins called phosphatonins, has been reported to be closely associated with cardiovascular risks, left ventricular hypertrophy, and vascular calcification.[95-97] FGF23 is linked to phosphate excretion. FGF23, similar to PTH, is released with high serum phosphate. Although PTH and FGF23 act synergistically on the proximal tubular epithelial cells, limiting phosphate reabsorption (i.e., increasing phosphaturia), their effects in other pathways are antagonistic. PTH stimulates renal activation of vitamin D (calcitriol), leading to increased intestinal absorption of calcium and phosphate. In contrast, FGF23 decreases calcitriol synthesis and stimulates its degradation, resulting in decreased intestinal absorption of calcium and phosphate.[98,99]

Levels of FGF23 and PTH increase with the severity of CKD. FGF23 starts to increase earlier than PTH in the course of CKD. Increased FGF23 is reported when GFR decreases from 90 to 60 mL/min per 1.73 m2; increasing significantly more with decreasing GFR. Changes in calcitriol follow FGF23, decreasing when GFR falls below 60-70 mL/min per 1.73 m2. Finally, PTH increases as the GFR falls below 45 and 50 mL/min per 1.73 m2. Increased serum phosphate is observed as GFR falls below 40 mL/min per 1.73 m^2.[100] This sequence of events

indicates the efficacy of phosphaturic agents in elimination of phosphate via the kidney. These agents significantly increase single nephron phosphaturia which is sufficient to keep a normal serum phosphate level despite progressive loss of the total nephron number.[101]

8. MONOCYTE/MACROPHAGE ROLE

Monocytes are generally subtyped into 3 subsets via their surface markers: 1) classical: CD14+, CD16-, CCR2+ (MCP-1 receptor, monocyte chemoattractant protein); 2) Intermediate: CD14+, CD16+, CCR2+, 3) Non-classical: CD14+, CD16+, CCR2-. The classical monocyte's primary function is phagocytosis and cytokine production. The primary function of the intermediate monocyte is angiogenesis, while the primary function of the non-classical monocyte is collagen deposition anti-inflammatory effects.[102] Ziegler-Heitbrock et al. showed that CD14+CD16+ monocytes have some features common with mature tissue macrophages.[103] Although there are 3 monocyte subsets currently acknowledged, the majority of studies only refer to 2 monocyte subpopulations (i.e., CD14+CD16- and CD14+CD16+ monocytes) without further subdivision of the CD16+ cells.[102] The CD16+ monocytes represent less than 15% of the circulating monocyte population in healthy humans, Their proportions are increased in patients with stenotic coronary artery disease, and myocardial infarction due to the up-regulation of inflammatory cytokines.[104] These data may indicate a possible role of CD16+ monocytes for the advanced inflammation-mediated arteriogenesis/intraplaque angiogenesis.[102]

The various subsets of monocytes may function differently in plaque development.[105] A significant number of circulating endothelial progenitor cells (EPC) are of monocyte origin.[106] Monocytes in culture have been shown to develop endothelial cell phenotypes. Monocyte have been shown to participate in improving re-endothelialization after arterial injury.[102]

Similarly, macrophages are often divided into subsets based on function termed M1 or classically activated and M2 or alternatively activated macrophages. It is generally acknowledged that this *in vitro* typing overly simplifies the *in vivo* situation. M1 macrophages are characterized by high levels of pro-inflammatory cytokines and their production of reactive oxygen and nitrogen species. In contrast, M2 macrophages are described as predominantly anti-inflammatory and are associated with wound healing and tumor growth.[107]

There appears to be a reciprocal relationship between the differentiation of M1 and M2 macrophages and their requirement for fatty acid synthesis (FAS) and fatty acid oxidation (FAO)

respectively. Inflammatory signals including LPS and IFNγ, that are required to generate M1 macrophages, have been shown to drive FAS,[108] while the inhibition of inflammatory signals required for the differentiation of M2 macrophages involves FAO.[109-111] Cellular longevity is also thought to be supported by FAO.[112] The IL4 induced M2 phenotype is dependent upon signal transducer and activator of transcription 6 (STAT6) and the peroxisome proliferator-activated receptor γ (PPARγ) and its coactivator 1b (PGC1b).[113,114] Inhibition of FAO, prevents M2 activation and overexpression of PGC1b prevents an M1 response following stimulation with LPS and IFNγ.[58,113] The source of the fatty acids for FAO in M2 macrophages is from uptake via the CD36 receptor (which is induced by IL4) and subsequent lysosomal lipolysis, which is mediated by lysosomal acid lipase (inducible by IL4).[107]

Atherosclerosis is a chronic inflammatory process that is characterized by the development of lipid-rich plaques located in the artery walls. The formation of the plaque occurs first with lipid accumulation, then monocyte infiltration and then the lipid core formation.[115] Monocytes adhere to artery endothelial walls, migrate into the wall, mainly intimal layer, and then accumulate lipids leading to protrusion of the plaque into the arterial lumen. Although, the earliest detectable change is intimal thickening,[116] pathological neovascularization is evident in early and late stages of atherosclerosis.[117] Monocyte-derived macrophages play a pivotal role in lipid deposition and progression of atherosclerosis and in the development of new vessels.[118]

Neovascularization is a key component of atherosclerosis, and it is impacted by local ischemia and inflammation, both local and systemic.[102] The degree of neovascularization is associated with intima-media thickness.[119] Thickening of the intima impairs the flow of oxygen and nutrients. Low oxygen increases hypoxia inducible factor (HIF-1) leading to the promotion of vascular endothelial growth factor (VEGF) production,[120] which mobilizes EPCs,[121] a large proportion of which are of monocyte origin.[102,106]

In the ischemic environment, HIF-1α, escapes degradation due to the down-regulation of its transcription and its ability to bind factors like HIF-1β that activate key genes in angiogenesis. Other growth factors like platelet-derived growth factor and basic fibroblast growth factor (bFGF) are similarly regulated through hypoxia. Monocytes are attracted by the macrophage and T-lymphocyte derived VEGF, which stimulates endothelial cells to produce monocyte chemoattractant protein (MCP-1).[102]

Increased shear stress in areas of atherogenic lesions stimulate endothelial cells on vascular walls triggers expression of MCP-1 involved in inducing transcription factors to help mitigate the stress. This sequence of events in the presence of high cholesterol can trigger the expression of adhesion molecules like P-selection, E-selectin, VCAM-1, ICAM-1, and MCP-1 in endothelial cells and the activation of genes responsible for expression of CCR2 (MCP-1 receptor) in monocytes.[102,105,122]

When the intima of arterial walls is ischemic, monocytes are recruited through an integrin receptor, macrophage adhesion ligand (MAC-1), that interacts with endothelial adhesion molecules.[123,124] Monocyte recruitment is mostly at endothelial tight junctures facilitating monocyte entry into subendothelial space. Macrophages express VEGF, activating endothelial cell production of MCP-1, which increases permeability of the endothelial layer. Monocyte maturation to macrophages is accompanied by production of cytokines and growth factors.[102,123,125]

Age related changes in the cardiorenal systems mirror cardiovascular and renal changes with CVD and CKD.[31] Endothelial cells on the vascular walls of large arteries produce NO, an essential vasodilator. Aged endothelial cells exhibit a decline in endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production.[126,127] NO deficiency contributes to endothelial cell deterioration with age.[128] NO functions as an inhibitor of vascular inflammation, platelet aggregation, and aberrant cell proliferation. Thus, NO deficiency and inactivation results in significant vasomotor dysfunction and atherosclerotic vascular change.[31,129]

NO-deficient mice show increased mortality and shorter lifespans, supporting the favorable effects of NO to suppress aging. The expression of eNOS has been reported as both increased and decreased with age.[130] eNOS concentrations do not always correlate with the amount of NO, however. eNOS becomes uncoupled with aging and produces superoxide.[131] Oxidative stress in aging causes a functional inactivation of NO.[31]

Plaque-associated monocytes-macrophages help remodel their local environment. They express proteases, e.g., urokinase plasminogen activator[132] activating plasmin, and MMPs, which degrade collagen and proteoglycans in the extracellular matrix. Monocytes produce platelet-derived growth factor inducing mitotic activity of endothelial cells and vascular smooth muscle cells. Monocytes/macrophages ingest oxidized lipids and become foam cells. Vascular

remodeling is promoted by foam cells via stimulation of smooth muscle cell migration followed by a shift in endothelial function.[133] Once monocytes infiltrate into the intima, a portion of them differentiate into dendritic cells triggering the activation of antigen specific T lymphocytes and creating a local inflammatory environment.[102]

Monocytes produce pro-inflammatory molecules such as IL-6 and TNFα, a process mediated by stimulation of Toll-like receptors (TLR4) in activated monocytes.[134] Endothelial cells and monocytes generate an inflammatory cascade through the interaction of CD14, a monocyte endotoxin receptor and a co-receptor (TLR) and leading to monocytic activation.[135] Monocyte activation subsequently enhances the affinity of monocyte ligands to adhesion molecules and promotes monocyte–endothelium adhesion.[135] This has been demonstrated by the presence of micro-vessels within lipid-rich plaques strongly expressing adhesion molecules (ICAM-1, VCAM-1) thereby facilitating transendothelial migration of inflammatory cells (i.e., monocytes) into the plaque microenvironment.[136] This implicates the potential involvement of monocytes and their role in plaque neovascularization and plaque rupture.[102]

Inflammation also induces the production fibrogenic cytokines and growth factors, leading to fibrosis.[137] The accumulation of fibrillar collagens leads to irreversible dysfunction and results in heart failure and end-stage renal failure.[137-139] Although matrix metalloproteinases (MMPs) are traditionally believed to suppress fibrosis because of their proteolytic activity, MMPs, particularly MMP-9, play key roles in stimulating fibrillar extracellular matrix (ECM) collagen accumulation.[31,140,141]

There are 25 identified members of mammalian MMPs, which are classified into collegenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs.[142] MMPs are endogenously inhibited by the tissue inhibitors of metalloproteinases (TIMPs: TIMP1–4). All TIMPs inhibit MMPs with relatively low selectivity by forming tight non-covalent 1:1 complexes. MMPs are important key regulators of ECM turnover by remodeling and degrading ECM components.[31]

Macrophages take up LDL, VLDL and oxidized lipoproteins via macropinocytosis, phagocytosis and scavenger receptor-mediated pathways including LOX-1, SR-A1, CD36 and SR- B1. Free cholesterol and fatty acids are generated following degradation of ingested lipids in the lysosome. Accumulation of cellular cholesterol leads to activation of several transcription

factors (TFs) including the liver X receptor α and β (Lxra, Lxrb), the retinoid x receptor (Rxr) and members of the PPAR family including PPARA and PPARG.[143,144] Alternatively, passive efflux of free cholesterol can also occur.[107]

These scavenger receptors do not only function in lipid uptake. For example, SR- A1 has also been shown to modulate macrophage proliferation, thereby regulating macrophage numbers.[145] CD36 has been implicated in inflammasome activation[146] and macrophage polarization.[147] Further, LOX-1, CD36, and SR-A1 act as apoptotic cell receptors, required for removal of cells, which have undergone apoptosis, by macrophages (a process called efferocytosis).[107,148-151]

Free cholesterol is toxic to cells therefore it is generally transported to the endoplasmic reticulum (ER) where it is re-esterified to form cholesterol esters that can be stored as relatively inert material in lipid droplets exported out of the cell via transporters such as ABCA1. Excess free cholesterol in the ER, can result in defective re-esterification increasing accumulation of free cholesterol in the cell, e.g., as lipid rafts in the membrane.[152] This causes enhanced inflammatory signaling at the membrane lipid rafts including TLR signaling and NF-KB activation.[152] Inflammatory signaling is further exacerbated if cholesterol transport from lysosomes becomes defective. These phenomena contribute to increased ER stress in the macrophages which results in cell death by apoptosis.[153] Apoptotic macrophages are cleared by other local macrophages by efferocytosis. However, this requires intact lipid metabolism. In atherosclerosis, the dysregulated lipid metabolism in macrophages prevents effective efferocytosis and this coupled with the increase in macrophage apoptosis results in secondary necrosis.[154] One mechanism that can prevent efferocytosis is cleavage of an apoptotic receptor MerTK from macrophages by ADAM17.[155] The subsequent release of intracellular components leads to necrosis, which is generally found in advanced plaques.[107]

The main function of LXRs is as cholesterol sensors, regulating gene expression in response to specific oxysterol ligands.[156,157] In macrophages, such oxysterols may be derived from internalized oxLDL or generated intracellularly through modification of cholesterol.[158,159] One of the best-known functions of LXRs is in the regulation of reverse cholesterol transport. In macrophages, LXRs reduce cellular cholesterol levels by regulating the expression of the cholesterol efflux transporters ABCA1 and ABCG1.[156] In addition to inducing expression of cholesterol efflux transporters, LXR signaling can induce the expression of apolipoproteins such as ApoE and ApoC which serve as receptors for cholesterol.[160] The importance of

apolipoproteins in macrophage cholesterol efflux is evident as mice lacking ApoE specifically in macrophages are more susceptible to atherosclerosis.[161] Increased production of lipoprotein remodeling proteins including LPL is a further mechanism by which LXRs function in macrophages to reduce the cholesterol burden.[162] Finally, it has also been reported that LXR induced expression of Idol in macrophages can induce LDLR ubiquitination leading to its degradation via the proteasome and subsequently reduced LDL cholesterol binding and uptake.[163] In addition to reducing cholesterol levels, several studies have also implicated LXRs in the modulation of glucose metabolism and in innate and adaptive immune responses. In macrophages, the latter equates to LXR activation limiting the production of inflammatory mediators such as iNOS and Cox2.[107,164]

Functionally, PPARγ has been suggested to control the inflammatory potential of macrophages being implicated in driving M2 polarization of macrophages as well as inhibiting pro-inflammatory gene expression including IL1 β, IL6, TNF α, IL12 and iNOS.[114,165,166] However, more recently PPARγ has also been shown to be required for uptake of apoptotic cells by macrophages,[165] providing an alternative mechanism through which PPARγ in macrophages contributes to the resolution of inflammation. In addition, perhaps the best-known role of the fatty acid receptor, PPARγ, is in cholesterol metabolism. PPARγ has been shown to regulate lipid accumulation in macrophages in atherosclerotic plaques. It achieves this by regulating expression of the scavenger receptors involved in lipid uptake, CD36 and SR-A,[167] as well as (through activating LXRα) regulating expression of cholesterol efflux genes including the transporters ABCA1 and ABCG1.[168] As a result, low or absent expression of PPARγ in macrophages is associated with increased atherosclerosis[168] and insulin resistance.[107,114]

III. LITERATURE REVIEW

Chronic kidney disease (CKD) is a major cause of morbidity and mortality in the US. The prevalence of CKD currently is about 14%, or about 26 million people, and the number of deaths from CKD has doubled over the past 20 years.[1,2] A significant number of CKD patients demonstrate accelerated cardiovascular disease (CVD) with meta-analysis suggesting an increased mortality of 1.4–3.7 times from CVD among patients with CKD.[2,7] In ESRD patients, who are dialysis-dependent, the risk of cardiovascular mortality is 10-fold to 20-fold higher than in patients without CKD.[8,9] Kidney disease and CVD are interrelated, in that disease in one system generates dysfunction in the other, with accelerating disease progression in both.[10] The interconnectedness of CKD and CVD is largely due to clustering of risk factors for CKD, including anemia, volume overload, abnormal mineral metabolism, proteinuria, oxidative stress, and inflammation, and risk factors for CVD, including age, hypertension, diabetes, and dyslipidemia. The hazard ratio for cardiovascular events increases progressively from 1.0 at GFR >60 to 3.4 at a GFR <15.[10] There are several theories for the increased risk of CVD in CKD, including increased oxidative stress, inflammation, platelet dysfunction, accelerated atherosclerosis, and reduced responsiveness to anti-platelet agents.[13-17] Heart disease primarily presents in two ways in patients with CKD: atherosclerotic vascular disease, especially coronary artery disease (CAD), and LVH. According to Gross and Ritz, the primary role of arteriosclerosis in left ventricular hypertrophy (LVH) is its contribution to central artery stiffness. A recent interventional study demonstrated the importance of arterial stiffness as a determinant of LVH by suggesting that alteration of aortic stiffness was the principal factor responsible for longitudinal changes in LV mass.[28] Atherosclerosis is progressive with reduced GFR.[20,21] Stiffening is accomplished through remodeling of these arteries by hypertrophy of both the intima and the media, breaks and disordered bedding of elastic fibers, as well as calcification. Similar changes in arteriosclerosis are seen with aging and are characterized by diffuse over-stretching of arteries, stiffening of the central arteries, and increased calcium deposits in the media layer.[28] Arterial stiffness of ageing arterial media is associated with increased expression of matrix metalloproteinases (MMP), which are members of the zinc-dependent endopeptidase family. One of their functions is degradation of vascular elastin and collagen fibers. Although there are numerous types of MMPs, MMP2 and MMP9 are the most studied in relation to arterial stiffness.[48]

Monocytes are part of the innate immunity, best known for their roles in the initiation and termination of inflammation. The influx of monocytes into vessel walls is a key process underlying the initiation of atherosclerosis.[169] When the intima of arterial walls is ischemic, monocytes are recruited through an integrin receptor, macrophage adhesion ligand (MAC-1), that interacts with endothelial adhesion molecules.[123,124] Monocyte recruitment is mostly at endothelial tight junctures facilitating monocyte entry into subendothelial space. Macrophages express VEGF, activating endothelial cell production of MCP-1, which increases permeability of the endothelial layer. Monocyte maturation to macrophages is accompanied by production of cytokines and growth factors.[102,123,125] Plaque-associated monocytes-macrophages help remodel their local environment. They express proteases, e.g., urokinase plasminogen activator[132] activating plasmin, and MMPs, which degrade collagen and proteoglycans in the extracellular matrix. Monocytes produce platelet-derived growth factor inducing mitotic activity of endothelial cells and vascular smooth muscle cells. Monocytes/macrophages ingest oxidized lipids and become foam cells. Vascular remodeling is promoted by foam cells via stimulation of smooth muscle cell migration followed by a shift in endothelial function.[133] Once monocytes infiltrate into the intima, a portion of them differentiate into dendritic cells triggering the activation of antigen specific T lymphocytes and creating a local inflammatory environment.[102] Monocyte-derived macrophages play a pivotal role in lipid deposition and progression of atherosclerosis and in the development of new vessels.[118]

The current pilot study undertook to examine transcription differences in monocytes from subjects in the early stages of CKD compared to Healthy control with focus on transcriptional activity of genes related to CKD and CVD. Using a model of early CKD provides the opportunity to identify areas of potential therapeutic intervention.

IV. METHODS

1. SUBJECTS

The study was approved by the Human Subjects Division of the University of Washington IRB from 2004 and is currently ongoing. Subjects were recruited from a larger Seattle Kidney Study and signed consent forms to undergo blood draw and monocyte isolation. Three case subjects and three Healthy controls were recruited. Case subjects were recruited from local renal clinics. Monocytes isolated from three pre-dialysis adult case subjects with chronic kidney disease and three matched controls. Chronic kidney disease was defined either as an estimated glomerular filtration rate <90 ml/min/1.73m^2 or albuminuria tested by either a spot ratio of ≥30 mg/g albumin to creatinine or a 24h collection of 30 mg albumin/24 hours. By self-report, Controls did not have chronic kidney disease.

Vitamin D is ubiquitously prescribed in CKD and was allowed, as were statins and anti-hypertensive agents. Controls were non-diabetic, not using bone-related medications, and matched to CKD subjects according to age (±3 years) and sex.

2. MONOCYTE ISOLATION

Monocytes were isolated from the blood of pre-dialysis CKD patients and matched controls (n= 3/group) using a negative selection procedure. This method was required to avoid or reduce activation of the monocytes during the process by direct antibody binding to cell surface molecules such as CD14 or CD16. Thus, a negative isolation method from Miltenyi (Miltenyi Biotec) was used to enrich monocytes from the total peripheral blood mononuclear cell fraction (PBMC). PBMCs were isolated from peripheral blood using the BD Vacutainer Cell Preparation Tubes, which employ a Ficoll-Hypaque density gradient to separate blood cell fractions. The PBMCs were labeled with a non-monocyte depletion antibody cocktail (Miltenyi Biotec) to eliminate lymphocytes, CD15+ granulocytes, and CD56+ NK cells. The labeled cells were run over a MACS column (Miltenyi Biotec) that magnetically separated the non-monocytes while the flow through fraction contained unlabeled monocytes. Although highly variable, this procedure yielded about a million monocytes from each ml of blood. The isolated monocytes were placed in culture in the presence of the autologous uremic or control serum and allowed to adhere for 4 hours. Serum solution was 10% serum + 20 ng/ml macrophage colony stimulating factor (PeproTech #300-25 Lot#081285). Serum was diluted in Phenol-free RPMI-1640 cell

culture media + Penicillin/streptomycin. Monocytes were cultured for 12 h in serum-free media and the conditioned media and total RNA was collected for microarray analyses.

3. RNA ISOLATION AND QC

RNA was isolated from monocytes using the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's recommended protocol. Integrity of RNA samples was assessed with an Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). RNA integrity was assessed by observing distinct and sharp 18 s and 28 s ribosomal RNA peaks that were baseline separated. RNA quantity was determined by measuring OD260 with a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The NanoDrop instrument was also used to determine purity of RNA samples by measuring OD260/280 and OD260/230 ratios. Only samples with OD260/280 and OD260/230 ratios >1.8, high integrity (RIN >7) and sufficient quantity were processed further.

4. RNA MICROARRAYS: DATA GENERATION, AND VERIFICATION

Data from 12 preparations were analyzed: CKD subjects: 3 RNA preparations from freshly isolated monocytes, 3 RNA preparations from incubated monocyte in autologous serum; Healthy control subjects: 3 RNA preparations from freshly isolated monocyte, 3 RNA preparations from incubated monocyte in autologous serum. All microarrays were processed by the Functional Genomics Laboratory of the UW Interdisciplinary Center for Exposures, Diseases, Genomics & Environment.

Microarray processing methods for the Affymetrix Human Clariom S microarray platform: The Functional Genomics Core Laboratory followed the manufacturer's protocols for the technical aspects associated with the determination of gene expression using Affymetrix Human Clariom S arrays (ThermoFisher, Affymetrix, Waltham, MA). Hybridized Affymetrix arrays were scanned with an Affymetrix GeneChip® 3000 fluorescent scanner. Image generation and feature extraction was performed using Affymetrix GeneChip® Command Console® Software.

Prior to making any comparisons, data were checked to ensure there were no unforeseen problems. One of the best ways to do this is via a principal-components analysis (PCA) plot, which is simply a way to plot high dimensional data (data where thousands of observations are

available) so the overall structure of the data can be observed. Samples are generally expected to be either grouped together by subject, or by treatment, or by status, depending on which has a larger effect on gene expression.

Both the D4 CKD and the H3 Healthy samples are down-weighted, which is reflected in the PCA plot. Outlier data can either be excluded or minimized to negate any negative effects. With limited samples, a weighting scheme was chosen, which down-weighted the apparent outlier samples.

The raw data was summarized and normalized using the RMA algorithm,[170] as implemented in the Bioconductor oligo package,[171] and then genes were filtered out that did not appear to be expressed in all samples. A weighted analysis of variance (ANOVA) model was used in conjunction with empirical Bayes adjusted contrasts to make comparisons. This is very similar to fitting individual t-tests, but power is gained to detect true differences in three ways. First, by fitting an ANOVA model and computing contrasts, the denominator of our statistic is based on the intra-group variance over all groups, instead of just the two groups under consideration (as is the case for the t-test). This increases power because the sample variance is not an efficient statistic, meaning it requires more samples to converge towards the underlying parameter it is intended to estimate. By using all samples, the number of samples used is increased to make this estimate, thereby increasing the accuracy. Second, by using a weighted ANOVA, individual samples can be smoothly up or down-weighted based on similarity to other similar sample types. This minimizes the need to make decisions about removing possible outlier samples from consideration. This approach has been shown to increase power in both real and simulated studies.[172] Third, the empirical Bayes adjustment for the contrasts uses a chip-wide estimate of variance to adjust the variance estimates for each gene. The chip-wide estimate, being based on so much data, is likely to be more accurate than the by-gene estimates, and has been shown to increase accuracy in both real and simulated data sets as well.[173]

5. DATA PRESENTATION

Gene transcription data are presented using Ingenuity platform as gene groupings of diseases and biofunctions, as part of a canonical pathway, as a gene set analysis, and as individual genes. Genes that were grouped by the diseases and biofunctions are loose associations of genes. There are no a priori relationships between the genes regarding how they may be working together. In

contrast the canonical pathway grouping of the genes uses established metabolic pathways with relationships regarding how genes function to produce the pathway products. Both these gene groupings use a differential gene expression definition based on false discovery rates, chosen by the investigator. Genes were selected based on a false discovery rate (FDR) of 0.05 with Fisher's exact test, meaning there is at most an expected 5% of the selected genes to be false positives. In both the disease-biofunction groupings and canonical pathway groupings, z-score was used as an additional criterion to determine significance. Z-scores greater than 2.0 or less than -2.0 were considered significant. Gene set analysis used ranking based on t-test to determine significance. This provided an ability, in some cases, to detect smaller differences and also to estimate significance when there was no uniform direction of regulation, and thus mixed results. For example if a gene associated with a canonical pathway produced an inhibitor of an enzyme, then the up-regulation of the pathway would include down-regulation of the inhibitor.

V. RESULTS

1. OVERVIEW OF DIFFERENTIAL GENE TRANSCRIPTION

As shown in the Venn diagram in Figure 1, 57 genes were transcribed at a different rate in the fresh monocyte preparation, of which 22 were shared with genes that were transcribed differently during monocyte incubation. During the incubation of monocytes from CKD and Healthy subjects, 2565 genes were either up-regulated or down-regulated. In the context of this comparison, up-regulated refers to genes that were transcribed at a higher rate in CKD subjects' monocytes compared to gene transcription rates from Healthy subjects' monocytes. Differential gene transcription between incubated monocytes from CKD subjects and Healthy subjects was the focus of this investigation for 2 reasons: 1) incubated monocytes more accurately represent *in situ* circulating monocytes than freshly isolated monocytes, and 2) a large number of genes were differentially transcribed by incubated monocytes.

Figure 1: Venn Diagram of Differential Gene Transcription: CKD subjects vs Healthy controls

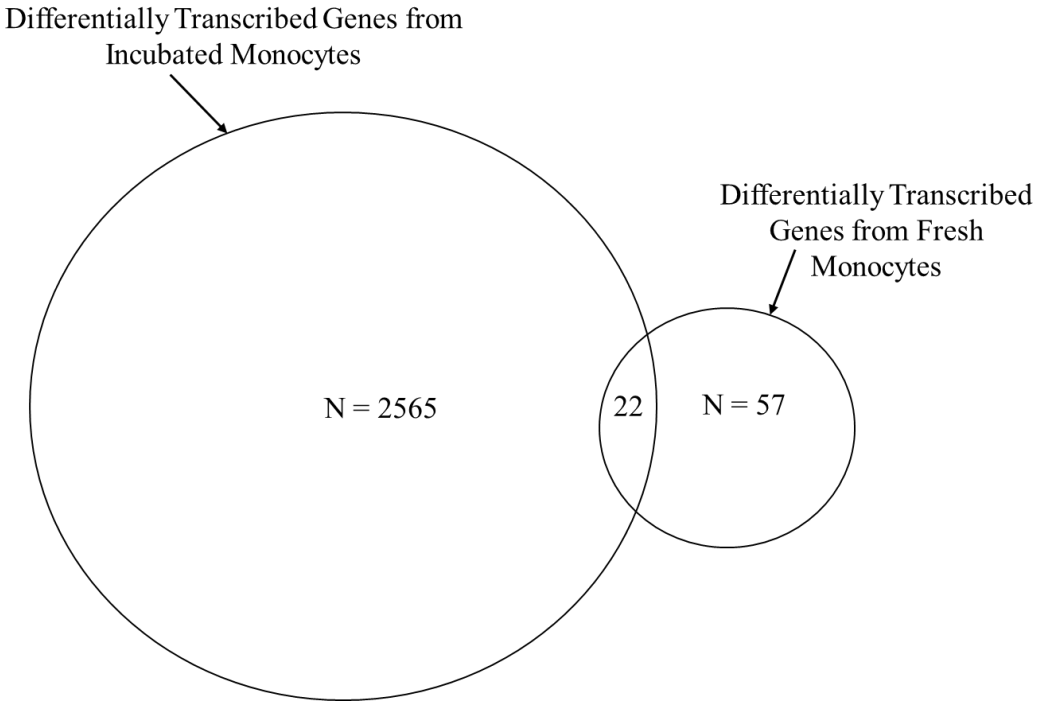


Table 2: Selected Diseases and Biofunctions, Highest Up-regulated Differential Transcription

Categories	Diseases or Functions	P-Value	Predicted	z-score	# Molecules
Cardiovascular System Development[1]	Permeability of vascular system	0.0000845	Increased	2.154	49
Cell Death and Survival	Cell survival	0.00000395	Increased	5.894	367
Cell Death and Survival	Cell viability	0.0000129	Increased	5.965	344
Cellular Compromise	Stress response of cells	0.0000000303	Increased	2.189	49
Cellular Compromise[1]	Endoplasmic reticulum stress response	0.000222	Increased	2.266	48
Cellular Development[1]	Proliferation of monocytes	0.000640	Increased	2.421	13
Cellular Movement	Cell movement of myeloid cells	0.00000118	Increased	2.028	161
Cellular Movement[1]	Cell movement of neutrophils	0.0000000284	Increased	2.157	100
Cellular Movement[1]	Cell movement of phagocytes	0.00000204	Increased	2.478	160
Cellular Movement[1]	Migration of neutrophils	0.000443	Increased	2.484	34
Connective Tissue Disorders[1]	Experimentally-induced arthritis	0.00000196	Increased	2.121	47
Lipid Metabolism[1]	Synthesis of lipid	0.000000532	Increased	2.685	192
Lipid Metabolism[1]	Metabolism of membrane lipid derivative	0.0000245	Increased	2.072	105
Lipid Metabolism[1]	Catabolism of lipid	0.000443	Increased	2.118	34

[1]Full category descriptions, alphabetically: "Cardiovascular System Development and Function, Tissue Morphology"; "Cellular Compromise, Cellular Function and Maintenance"; "Cellular Development, Cellular Growth and Proliferation, Embryonic Development, Hematological System Development and Function, Hematopoiesis, Lymphoid Tissue Structure and Development, Organ Development, Organismal Development, Tissue Development"; "Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response"; "Connective Tissue Disorders, Inflammatory Disease, Inflammatory Response, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders"; "Lipid Metabolism, Small Molecule Biochemistry"

The selection of up-regulated diseases and biofunctions shown in Table 2 are derived from 55 diseases and biofunctions that exhibited significantly different transcription between CKD subjects and Healthy controls. These diseases and biofunctions are groupings of genes that were significantly up-regulated or down-regulated and were associated with the disease or biofunction. Table 2 provides a listing of diseases and biofunctions deemed most pertinent to monocytes and kidney disease. Of the up-regulated diseases and biofunctions, cell survival and viability, stress response, cell movement (and inflammatory response), connective tissue disorder, and lipid metabolism are common themes. Table 3 lists down-regulated diseases and biofunctions, and indicating lower transcription in CKD subjects. Diseases and biofunctions associated with cell death and connective tissue were most pertinent to monocytes and kidney disease.

Table 3: Selected Diseases and Biofunctions with Highest Down-regulated Differential Transcription

Categories	Diseases or Functions	P-Value	Predicted	z-score	# Molecules
Cell Death and Survival	Necrosis	0.0000000148	Decreased	-3.815	646
Cell Death and Survival	Cell death	0.00000213	Decreased	-4.385	782
Cell Death and Survival	Apoptosis	0.000158	Decreased	-2.990	598
Connective Tissue Disorders[1]	Abnormal bone density	0.000311	Decreased	-2.563	56

[1]Full category description: "Connective Tissue Disorders, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders"

Canonical pathways are groupings of genes that function in defined pathways, and only genes with significant differential transcription were included. Table 4 lists all the canonical pathways that displayed significant differential transcription between CKD and Healthy subjects. Of the pathways, only the antioxidant action of vitamin C was down-regulated.

Table 4: Ingenuity Canonical Pathways with Largest Transcription Changes

Ingenuity Canonical Pathways	**p-value**	**z-score**
Superpathway of Cholesterol Biosynthesis	0.00000000209	4.243
Cholesterol Biosynthesis I	0.0000000562	3.317
NRF2-mediated Oxidative Stress Response	0.0000191	3.657
tRNA Charging	0.000646	3.742
Neuroinflammation Signaling Pathway	0.000977	2.474
Antioxidant Action of Vitamin C	0.00191	-2.558
TNFR1 Signaling	0.00309	2.138
Methionine Degradation I (to Homocysteine)	0.00437	2.828
Superpathway of Geranylgeranyldiphosphate Biosynthesis I	0.00603	2.646
CDP-diacylglycerol Biosynthesis I	0.00631	2.333
PI3K Signaling in B Lymphocytes	0.00832	3.157
Dendritic Cell Maturation	0.00851	2.197
Cysteine Biosynthesis III (mammalia)	0.00891	2.828
RANK Signaling in Osteoclasts	0.00933	3.545
iNOS Signaling	0.00933	2.309
B Cell Activating Factor Signaling	0.0107	2.714
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.0110	2.558
Phosphatidylglycerol Biosynthesis II (Non-plastidic)	0.0115	2.333
CD40 Signaling	0.0129	2.065
4-1BB Signaling in T Lymphocytes	0.0135	2.333
TCA Cycle II (Eukaryotic)	0.0162	2.121
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.0178	2.402
April Mediated Signaling	0.0186	2.111
ERK5 Signaling	0.0204	2.500
Mevalonate Pathway I	0.0251	2.236
Superpathway of Methionine Degradation	0.0257	3.162
Tec Kinase Signaling	0.0282	3.024
Oxidative Phosphorylation	0.0302	3.710
Role of IL-17F in Allergic Inflammatory Airway Diseases	0.0324	2.530
IL-8 Signaling	0.0380	4.564

Common themes in the canonical pathways were cholesterol metabolism (superpathway of cholesterol biosynthesis, cholesterol biosynthesis 1, superpathway of geranylgeranyldiphosphate biosynthesis I, mevalonate pathway I), lipid metabolism (CDP-diacylglycerol Biosynthesis I, Phosphatidylglycerol Biosynthesis II), and oxidation and inflammation (NRF2-mediated

Oxidative Stress Responses, Neuroinflammation Signaling Pathway, Antioxidant Action of Vitamin C, TNFR1 Signaling, and Production of Nitric Oxide and Reactive Oxygen Species in Macrophages).

For each canonical pathway, a set of genes were included, and many of the pathways overlapped individual genes. Analyzing which genes were most common in these pathways helped focus attention to where important regulation occurs. Table 5 displays the top 10 genes in these pathways. With the exception of C3, all of the genes identified are involved in RANK signaling and NF-KB activation.

Table 5: Common Genes involved in Significant Canonical Pathways

Symbol	Gene Name	# Pathways
NFKB1	nuclear factor kappa B subunit 1	20
NFKB2	nuclear factor kappa B subunit 2	19
C3	complement C3	15
CHUK	conserved helix-loop-helix ubiquitous kinase	15
IKBKB	inhibitor of nuclear factor kappa B kinase subunit beta	15
IKBKE	inhibitor of nuclear factor kappa B kinase subunit epsilon	15
NFKBIE	NFKB inhibitor epsilon	14
ATM	ATM serine/threonine kinase	10
PIK3R1	protein phosphatase 3 regulatory subunit B, alpha	9
PIK3C3	phosphatidylinositol 3-kinase catalytic subunit type 3	8

Data presented in the overview of differential transcription provided the rationale for focusing on cholesterol metabolism, lipid metabolism, and oxidation-inflammation with emphasis on cell death and survival, RANK signaling of NF-KB, and connective tissue.

2. CHOLESTEROL

Genes involved in the cellular influx and efflux of cholesterol are shown in Table 6. Low density lipoprotein receptor (LDLR), the primary mechanism for influx of cholesterol, was significantly up-regulated. In contrast, all of the genes involving cholesterol efflux were down-regulated. With the exception of MARCO, genes involved in the uptake of oxidized LDL and other oxidized lipoproteins (MARCO, CD36, OLR1, SRA1) were down-regulated. Receptors from these genes were also involved in the recognition and phagocytosis of apoptotic cells.

Table 6: Cholesterol Influx and Efflux from Cells

Symbol	Entrez Gene Name	Log-fold Change	P-Value	Transport	Regulation
LDLR	low density lipoprotein receptor	1.415767445	0.0000351	Influx	Upregulation
MARCO	macrophage receptor with collagenous structure	0.512210018	0.0326	Influx	Upregulation
CD36	CD36 molecule	-1.304054861	0.001126	influx	Downregulation
OLR1	oxidized low density lipoprotein receptor 1	-1.386590111	0.000305	Influx	Downregulation
TLR4	toll like receptor 4	-1.622548431	0.00000479	Influx	Downregulation
SRA1	steroid receptor RNA activator 1	0.0363925	0.864	Influx	No change
LRP1	LDL receptor related protein 1	-0.107710568	0.597	Influx	No change
APOE	apolipoprotein E	-0.797900095	0.00141	Efflux	Downregulation
ABCA1	ATP binding cassette subfamily A member 1	-0.669490099	0.00744	Efflux	Downregulation
ABCG1	ATP binding cassette subfamily G member 1	-0.717640929	0.00167	Efflux	Downregulation
SCARB1	scavenger receptor class B member 1	-0.505586042	0.0353	Efflux	Downregulation

The superpathway of cholesterol biosynthesis includes; cholesterol bioynthesis pathways I, II, and III. The superpathway (collectively) and the individual cholesterol biosynthesis pathways were significantly up-regulated (data shown for biosynthesis I). Genes from the cholesterol pathways were the most significantly up-regulated among all of the canonical pathways in the comparison of CKD subjects and Healthy controls, as shown in Table 7.

Table 7: Genes Differentially Expressed in Cholesterol Biosynthesis

Symbol	Entrez Gene Name	Log-fold Change	P-Value	Location	Type
MSMO1	methylsterol monooxygenase 1	2.634	0.000000101	Cytoplasm	enzyme
CYP51A1	cytochrome P450 family 51 subfamily A member 1	2.619	0.000000604	Cytoplasm	enzyme
FDFT1	farnesyl-diphosphate farnesyltransferase 1	1.758	0.000000981	Cytoplasm	enzyme
IDI1	isopentenyl-diphosphate delta isomerase 1	2.105	0.00000109	Cytoplasm	enzyme
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	2.259	0.00000228	Cytoplasm	enzyme
DHCR7	7-dehydrocholesterol reductase	1.685	0.00000266	Cytoplasm	enzyme
SC5D	sterol-C5-desaturase	1.905	0.00000293	Cytoplasm	enzyme
FDPS	farnesyl diphosphate synthase	1.514	0.00000541	Cytoplasm	enzyme
SQLE	squalene epoxidase	3.055	0.0000133	Cytoplasm	enzyme
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	1.549	0.0000259	Cytoplasm	enzyme
DHCR24	24-dehydrocholesterol reductase	1.615	0.0000716	Cytoplasm	enzyme
ACAT2	acetyl-CoA acetyltransferase 2	1.444	0.000340	Cytoplasm	enzyme
GGPS1	geranylgeranyl diphosphate synthase 1	0.798	0.000508	Cytoplasm	enzyme
HSD17B7	hydroxysteroid 17-beta dehydrogenase 7	0.821	0.00105	Cytoplasm	enzyme
NSDHL	NAD(P) dependent steroid dehydrogenase-like	0.565	0.00366	Cytoplasm	enzyme
EBP	EBP, cholestenol delta-isomerase	0.943	0.00396	Cytoplasm	enzyme
MVK	mevalonate kinase	0.624	0.00428	Cytoplasm	kinase
LBR	lamin B receptor	0.691	0.00112	Nucleus	enzyme

Similarly, the initial steps of the superpathway of geranylgeranyldiphosphate biosynthesis I and the mevalonate pathway I are contained in the superpathway of cholesterol biosynthesis.

These pathways were significantly up-regulated as well in CKD subjects. Transcription of genes from the mevalonate biosynthesis pathway that were significantly different between CKD and Healthy subjects were contained within the superpathway of cholesterol biosynthesis. Genes that displayed significantly differential transcription between groups are shown in Table 8.

Table 8: Genes Differentially Expressed Superpathway of Geranylgeranyldiphosphate Biosynthesis

Symbol	Entrez Gene Name	Log-fold Change	P-Value	Location	Type
IDI1	isopentenyl-diphosphate delta isomerase 1	2.105	0.00000109	Cytoplasm	enzyme
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	2.259	0.00000228	Cytoplasm	enzyme
FDPS	farnesyl diphosphate synthase	1.514	0.00000541	Cytoplasm	enzyme
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	1.549	0.0000259	Cytoplasm	enzyme
ACAT2	acetyl-CoA acetyltransferase 2	1.444	0.00034	Cytoplasm	enzyme
GGPS1	geranylgeranyl diphosphate synthase 1	0.798	0.000508	Cytoplasm	enzyme
MVK	mevalonate kinase	0.624	0.00428	Cytoplasm	kinase

3. LIPIDS

Diseases and biofunctions of lipids are shown in Table 9. Genes associated with both the synthesis and catabolism of lipids were significantly up-regulated in CKD subjects, as were genes associated with membrane lipid metabolism.

Table 9: Lipid-Related Diseases and Biofunctions Differentially Expressed

Categories	Functions	Diseases or Functions	p-Value	Predicted	z-score	Molecules
Lipid Metabolism	Synthesis	Synthesis of lipid	0.000000532	Increased	2.685	192
Lipid Metabolism	Metabolism	Metabolism of membrane lipid	0.0000245	Increased	2.072	105
Lipid Metabolism	Catabolism	Catabolism of lipid	0.000443	Increased	2.118	34

CDP-diacylglycerol biosynthesis I pathway and phosphatidylglycerol biosynthesis II are both up-regulated as part of phospholipid biosynthesis, and in this study CDP-diacylglycerol synthase 2 (CDS2) was up-regulated as a key regulatory step (p-value: 0.003), as shown in Table 10. The initial enzymes were all up-regulated in CKD subjects. Phosphatidylcholine synthesis (lysophosphatidylcholine acyltransferase 3 p-value: 0.0006), however, was down-regulated.

Table 10: Genes Differentially Expressed in CDP-diacylglycerol Biosynthesis I and Phosphatidylglycerol Biosynthesis II

Symbol	Entrez Gene Name	Log-fold Change	P-value	Location	Type(s)
LPCAT3	lysophosphatidylcholine acyltransferase 3	-0.792	0.000619	Plasma Membrane	enzyme
MBOAT7	membrane bound O-acyltransferase domain containing 7	0.615	0.00397	Plasma Membrane	enzyme
AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5	1.493	0.00000603	Cytoplasm	enzyme
CRLS1	cardiolipin synthase 1	0.845	0.000283	Cytoplasm	enzyme
CDS2	CDP-diacylglycerol synthase 2	0.989	0.000327	Cytoplasm	enzyme
GPAT4	glycerol-3-phosphate acyltransferase 4	0.566	0.00282	Cytoplasm	enzyme
AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	0.601	0.00424	Cytoplasm	enzyme
MBOAT2	membrane bound O-acyltransferase domain containing 2	0.908	0.00511	Cytoplasm	enzyme
MBOAT1	membrane bound O-acyltransferase domain containing 1	1.002	0.00568	Cytoplasm	enzyme

The canonical pathways of triacylglycerol biosynthesis (p-value: 0.271) and degradation (p-value: 0.171) were neither up-regulated nor down-regulated.

Of the 128 genes identified as associated with triglyceride metabolism, 29 genes had significantly different transcription between CKD subjects and Healthy controls. The coordinated regulation of TG and phosphatidylcholine synthesis is through phosphatidic acid phosphohydrolase (PAP) (gene coding PLPP) and CTP phosphocholine cytidylyltransferase (gene coding: PCYT1A). Both PLPP3 (p-value: 0.0237) and PLPP7 (p-value: 0. 0.0403) were significantly stimulated for CKD and Healthy subjects. Of the 2 forms of PCYT1 (A and B), PCYT1 Isoform A was up-regulated in CKD subjects.

Gene set analysis was also performed on genes that were specifically related to synthesis and degradation of triacylglycerol (Table 11). Gene set analysis allows for comparison of gene transcription unrelated to direction of regulation. Similar to canonical pathways analysis, directional regulation was not significant in gene set analysis. When direction was removed as a parameter, however, a significant differential transcription between CKD and Healthy subjects became apparent (Table 9: FDR Mixed Biosynthesis: p-value: 0.00000302; Degradation, p-value: 0.000000482).

Table 11: Gene Set Analysis

	Genes	Direction	P-Value	FDR	P-value Mixed	FDR Mixed
Triacylglycerol Biosynthesis	44	Up	0.4560	0.5700	0.000003020	0.000003020
Triacylglycerol Degradation	37	Down	0.6510	0.6510	0.000000344	0.000000482

Data from canonical pathways indicated that there was no significant difference in gene transcription for either TAG synthesis or degradation. Gene set analysis indicated that there was no stimulation with one direction for either synthesis or degradation, however, there was

significant stimulation for both synthesis and degradation with the mixed directions. There was no uniform increase in direction. Also, there was significant stimulation of either lipid synthesis or lipid degradation in the gene function analysis.

The extracellular components of triglyceride metabolism that were up-regulated included Complement C3 and TNFSF10 (Table 12). In contrast, other extracellular proteins' genes were down-regulated, specifically apolipoproteins B, E, and C2. LDLR was the only CKD plasma membrane receptor gene that was up-regulated significantly. Several glutamate ionotropic receptors, as well as CD36 and perilipin 2, were down-regulated.

Table 12: Genes Differentially Expressed in Triglyceride Metabolism

Symbol	Entrez Gene Name	Log-fold Change	Expr p-value	Location	Family
C3	complement C3	2.525	0.0000000941	Extracellular Space	peptidase
APOB	apolipoprotein B	-1.136	0.0000523	Extracellular Space	transporter
APOE	apolipoprotein E	-0.798	0.00141	Extracellular Space	transporter
PRL	prolactin	-1.063	0.00147	Extracellular Space	cytokine
TNFSF10	TNF superfamily member 10	2.548	0.00233	Extracellular Space	cytokine
APOC2	apolipoprotein C2	-0.613	0.00540	Extracellular Space	transporter
PLIN2	perilipin 2	-3.332	0.0000000954	Plasma Membrane	other
LDLR	low density lipoprotein receptor	1.416	0.0000351	Plasma Membrane	transporter
GRIA4	glutamate ionotropic receptor AMPA type subunit 4	-0.758	0.000171	Plasma Membrane	ion channel
CD36	CD36 molecule	-1.304	0.00113	Plasma Membrane	transmembrane receptor
GRIA2	glutamate ionotropic receptor AMPA type subunit 2	-0.699	0.00580	Plasma Membrane	ion channel
ACSL1	acyl-CoA synthetase long chain family member 1	2.346	0.000000112	Cytoplasm	enzyme
DBI	diazepam binding inhibitor, acyl-CoA binding protein	1.243	0.0000933	Cytoplasm	other
INSIG1	insulin induced gene 1	1.129	0.000144	Cytoplasm	other
ITPR1	inositol 1,4,5-trisphosphate receptor type 1	1.056	0.000370	Cytoplasm	ion channel
CPT1A	carnitine palmitoyltransferase 1A	-0.983	0.000504	Cytoplasm	enzyme
INSIG2	insulin induced gene 2	0.708	0.00167	Cytoplasm	other
CAT	catalase	-1.093	0.00231	Cytoplasm	enzyme
GPAT4	glycerol-3-phosphate acyltransferase 4	0.566	0.00282	Cytoplasm	enzyme
LPL	lipoprotein lipase	1.561	0.00309	Cytoplasm	enzyme
CALM1 (in	calmodulin 1	0.791	0.00330	Cytoplasm	other
FABP1	fatty acid binding protein 1	-0.730	0.00353	Cytoplasm	transporter
ACSL5	acyl-CoA synthetase long chain family member 5	0.749	0.00481	Cytoplasm	enzyme
PNPLA3	patatin like phospholipase domain containing 3	-0.613	0.00597	Cytoplasm	enzyme
FOXO1	forkhead box O1	-1.367	0.000583	Nucleus	transcription regulator
PPARG	peroxisome proliferator activated receptor gamma	-1.103	0.00397	Nucleus	nuclear receptor

Regarding cytoplasmic components, up-regulated components included acyl-CoA synthetase 1 and 5, lipoprotein lipase (LPL), glycerol-3-phosphate acyltransferase 4, and inositol 1,4,5-trisphosphate receptor type 1. Down-regulated components in the mitochondria included carnitine palmitoyltransferase 1A and catalase.

Nuclear transcription regulators forkhead box O1 (FOXO1) and peroxisome proliferator activated receptor gamma (PPARG) were significantly down-regulated.

Although not listed as related to triacylglycerol metabolism, fatty acid binding protein (FABP 4), which is specifically expressed in adipose and macrophages, was significantly down-regulated (p-value: 0.0000746).

Regarding fatty acid metabolism, the canonical pathway analysis of FAO enzymes showed that there was no significant uniform increase or decrease in transcription rate of CKD subjects compared to Healthy subjects (p-value: 0.425). There is no similar canonical pathway analysis for FAS.

Gene analysis

Acetyl-CoA carboxylase (ACACA), a key regulator enzyme in the fatty acid synthesis (FAS) pathway, was significantly increased (Table 13). Fatty acid synthesis is transcriptionally regulated by sterol regulatory element-binding protein (SREBP – gene coding: SREBF1 and SREBF2) and carbohydrate responsive element binding protein (ChREBP – gene coding: MLXIPL). In this study, SREBF1 transcription in CKD subjects was not changed relative to Healthy controls (p-value: 0.086), whereas SREBF2 transcription was significantly increased (p-value: 0.016). Similarly, LXR (gene code: NR1H2 and NR1H3) transcription rate did not change via NR1H2 (p-value: 0.36) in CKD subjects versus Healthy controls, but did increase in isoform NR1H3 (p-value: 0.023). ChREBP transcription was significantly increased in CKD subjects relative to Healthy controls (p-value: 0.019).

Table 13: Key enzymes in Fatty Acid Metabolism

Pathway	**Gene**	**Log-fold Change**	**P-value**
FA synthesis			
Acetyl-CoA Carboxylase	ACACA	0.970	0.00199
Fatty Acid Synthase	FASN	0.291	0.120
Malonyl-CoA ACP Transacylase	MCAT	0.364	0.110
FA Oxidation			
Carnitine Palmitoyl Transferase 1a	CPT1a	-0.983	0.000504
Carnitine Palmitoyl Transferase 2	CPT2	0.186	0.306
Carnitine acylcarnitine translocase	SLC25A20	-0.455	0.0181

Transcription was decreased in key regulatory enzymes of the fatty acid oxidation (FAO) pathway. Transcription of carnitine palmitoyl transferase 1a (CPT1a) and carnitine acylcarnitine translocase (CACT), proteins responsible for facilitating metabolite transfer across the mitochondrial membrane, was decreased in CKD subjects versus Healthy controls.

4. RANK AND NF-KB

Pathway analysis

Transcription of genes in the RANK signaling pathway was estimated in bone metabolism using both Ingenuity Canonical Pathway analysis and Gene Set analysis (Table 14). Canonical pathway analysis using 24 molecules indicated that signaling was increased in CKD subjects compared to Healthy controls. Similarly, Gene Set Analysis using 99 molecules suggested that there was a significant increase in signaling in CKD subjects.

Table 14: Pathway Analysis for RANK Signaling in Osteoclasts

Analysis	Z-score	P-Value	Direction	Molecules
Ingenuity Canonical Pathway	3.545	0.00933	Increase	24
Gene Set Analysis Directional	-	0.0186	Increase	99
Gene Set Analysis Mixed	-	0.000000196	Mixed	99

Gene analysis

A majority of the genes in the RANK signaling pathway were differentially transcribed as shown in Table 15.

Table 15: Genes Differentially Expressed in RANK Signaling Pathway

Gene	Entrez Gene Name	Log-fold Change	P-value	Location	Type
GSN	gelsolin	2.029	0.0000000729	Extracellular Space	other
PPP3CB	protein phosphatase 3 catalytic subunit beta	0.739	0.000366	Plasma Membrane	phosphatase
MAP3K8	mitogen-activated protein kinase kinase kinase 8	-2.954	0.00000209	Cytoplasm	kinase
SRC	SRC proto-oncogene, non-receptor tyrosine kinase	1.638	0.00000447	Cytoplasm	kinase
IKBKE	inhibitor of nuclear factor kappa B kinase subunit epsilon	0.985	0.000149	Cytoplasm	kinase
BIRC2	baculoviral IAP repeat containing 2	0.713	0.000202	Cytoplasm	enzyme
CHUK	conserved helix-loop-helix ubiquitous kinase	0.842	0.00121	Cytoplasm	kinase
MAPK9	mitogen-activated protein kinase 9	1.109	0.00134	Cytoplasm	kinase
BIRC3	baculoviral IAP repeat containing 3	1.152	0.0016	Cytoplasm	enzyme
CALM1 (inclu	calmodulin 1	0.688	0.00194	Cytoplasm	other
CHP1	calcineurin like EF-hand protein 1	-0.957	0.0021	Cytoplasm	transporter
PIK3R1	phosphoinositide-3-kinase regulatory subunit 1	0.674	0.00227	Cytoplasm	kinase
MAP3K4	mitogen-activated protein kinase kinase kinase 4	0.683	0.00264	Cytoplasm	kinase
IKBKB	inhibitor of nuclear factor kappa B kinase subunit beta	0.720	0.0028	Cytoplasm	kinase
PPP3R1	protein phosphatase 3 regulatory subunit B, alpha	0.600	0.00353	Cytoplasm	phosphatase
MAPK13	mitogen-activated protein kinase 13	-0.647	0.00372	Cytoplasm	kinase
PIK3C3	phosphatidylinositol 3-kinase catalytic subunit type 3	0.530	0.00495	Cytoplasm	kinase
PPP3CC	protein phosphatase 3 catalytic subunit gamma	0.611	0.00497	Cytoplasm	phosphatase
MAP3K1	mitogen-activated protein kinase kinase kinase 1	0.508	0.00619	Cytoplasm	kinase
MAP3K3	mitogen-activated protein kinase kinase kinase 3	0.519	0.0065	Cytoplasm	kinase
CALM1 (inclu	calmodulin 1	-0.531	0.00726	Cytoplasm	other
NFKBIE	NFKB inhibitor epsilon	0.786	0.000165	Nucleus	transcription regulator
ATM	ATM serine/threonine kinase	0.947	0.00135	Nucleus	kinase
NFKB1	nuclear factor kappa B subunit 1	0.910	0.00179	Nucleus	transcription regulator
NFKB2	nuclear factor kappa B subunit 2	0.722	0.00362	Nucleus	transcription regulator

Differential transcription of RANK (gene coding: TNFRSF11A), RANKL (gene coding: TNFSF11), OPG (gene coding: TNFRSF11B), and TNF-Related Apoptosis Inducing Ligand

(TRAIL – gene coding: TNFSF10) are shown in Table 16. There were no significant changes in these key signaling components except for TRAIL. TRAIL was up-regulated significantly in CKD subjects (p-value: 0.002).

Table 16: RANK Signaling in Osteoclasts

Symbol	Entrez Gene Name	Log-fold Change	P-value	Location
TNFRSF11A	Receptor Activator of Nuclear Factor-Kappa B (RANK)	-0.340	0.101	Plasma Membrane
TNFSF11	Receptor Activator of Nuclear Factor-Kappa B Ligand (RANKL)	-0.291	0.301	Extracellular Space
TNFRSF11B	Osteoprotegerin (OPG)	-0.094	0.715	Extracellular Space
TNFSF10	TNF superfamily member 10, TRAIL	2.55	0.00233	Extracellular Space

5. Connective Tissue and MMP

The most significant differential gene transcription was of MMP9. This metalloproteinase is part of a family of MMPs, many of which were significantly down-regulated in CKD subjects. MMPs are inactivated by binding TIMPs. TIMP1 was significantly down-regulated (Table 17).

Table 17: Matrix Metalloproteinases

Symbol	Entrez Gene Name	Log fold change	p-value
MMP9	matrix metallopeptidase 9	3.833	0.0000000000349
MMP28	matrix metallopeptidase 28	-0.635	0.00312
MMP25-AS1	MMP25 antisense RNA 1	-0.554	0.00425
MMP2	matrix metallopeptidase 2	-0.758	0.00656
MMP20	matrix metallopeptidase 20	-0.600	0.00894
MMP19	matrix metallopeptidase 19	-1.246	0.0128
MMP1	matrix metallopeptidase 1	0.677	0.0154
MMP8	matrix metallopeptidase 8	0.968	0.0168
MMP16	matrix metallopeptidase 16	-0.477	0.0174
MMP11	matrix metallopeptidase 11	-0.533	0.0218
TIMP1	TIMP metallopeptidase inhibitor 1	-1.430	0.00171

VI. Discussion

Over 2500 genes were differentially transcribed in incubated monocytes from CKD subjects compared to matched Healthy controls as compared to only 57 differentially transcribed genes from non-incubated (fresh) monocytes. Monocytes are generally short-lived. They contribute to innate immunity through local recruitment where they differentiate into macrophages, dendritic cells, and endothelial cells.[174,175] Only three different monocyte subsets have been described that have different functions and half-lives.[176] Macrophages are more diverse and heterogeneous, with the ability to shift their functional state.[176-178] When monocytes bind to endothelial cells, genes are regulated that are required for the subsequent transendothelial migration (extravasation) step and the differentiation of monocytes into macrophages and dendritic cells is initiated. Thus, differential gene transcription of circulating (fresh) monocytes from CKD and Healthy controls were similar in our data because they represented generalized monocyte subsets, whereas differential gene transcription of incubated monocytes represented the initial stages of monocyte extravasation and differentiation into lineages that were more heterogeneous responding to serum components and to differentiation stimuli (e.g., macrophage colony stimulating factor).

When differentially transcribed genes were grouped according to diseases and biofunctions, cell survival and viability, immune cell movement and phagocytosis were up-regulated. Genes associated with monocytes proliferation were up-regulated in CKD subjects. Stress response of cells and particularly of genes associated with stress response in the endoplasmic reticulum, where cholesterol is processed, were up-regulated, suggesting increased oxidative damage, perhaps due to elevated cholesterol levels. In contrast, genes associated with cell death, necrosis, and apoptosis were down-regulated in CKD subjects.

When differentially transcribed genes were grouped into canonical pathways, up-regulation was most significant for genes associated with cholesterol metabolism (superpathway of cholesterol biosynthesis, cholesterol biosynthesis I, II, and III, superpathway of geranylgeranyldiphsophate biosynthesis, and mevalonate pathway I), with lipid metabolism (CDP-diacylglycerol biosynthesis I, phosphatidylglycerol biosynthesis II), and with oxidations and inflammation ((NRF2-mediated Oxidative Stress Responses, Neuroinflammation Signaling Pathway, Antioxidant Action of Vitamin C, TNFR1 Signaling, and Production of Nitric Oxide and Reactive Oxygen Species in Macrophages).

It is not surprising that genes associated with cell growth, inflammation, and oxidative damage were up-regulated, while genes associated with cell death were down-regulated. Monocytes were incubated in media, with little or no hypoxia, which would normally be a stimulant to recruit monocytes. It follows that there were significant anabolic processes. Canonical pathway data mirror diseases and biofunction data in suggesting CKD monocytes were anabolic, inflammatory, and under oxidative stress. Monocyte function is dependent on monocyte subset and local environment.[179] Circulating LDL and Lp(a) interact with monocytes and contribute to an enhanced inflammatory state. For example, monocytes from patients with familial hypercholesterolemia have increased expression of CD11b, CD18, and chemokines receptors CX3CR1 and CCR2.[180] Increase in CCR2 expression is correlated with elevated lipid accumulation in the monocytes and helps mediate monocytes toward vascular sites of inflammation.[169,180] Similarly, monocytes isolated from patients with familial dysbetalipoproteinemia, characterized by elevated plasma triglycerides and cholesterol, are phenotypically inflamed with increased expression of CD11b/CD11c/CD18.[181] In fact, the altered composition of monocytes derived from hypercholesterolemic patients results in different behavior, as shown by ex vivo increased transmigration capacity across endothelium.[169,180]

In order to understand where key regulatory differences occurred between monocytes from CKD and Healthy subjects, the genes most prevalent in all of the significant canonical pathways were examinied. The top most common genes are listed in Table 4. All of these genes, with the exception of C3, are part of RANK signaling of NF-KB, highlighting this pathway's prominence in CKD subject-related differences.

Genes associated with lipid synthesis and catabolism were significantly increased in CKD subjects compared to Healthy controls, suggesting that monocyte/macrophage lipid metabolism is handled differently in the environment of kidney disease. These lipid analyses included over 300 genes as well as membrane lipid metabolism. As part of the increased transcription, key regulatory enzymes of triglyceride and phosphatidylcholine synthesis were up-regulated. Both canonical pathway analysis of triglyceride synthesis and degradation and gene set analysis of these pathways in a specific direction revealed that there was no transcriptional increase or decrease between CKD subjects and matched Healthy controls. In contrast, gene set analysis of genes for both the synthesis and degradation of triglycerides were significantly different in CKD subject compared with Healthy controls, but with no uniform direction.

Transcription rate of the primary regulatory enzyme in the FAS pathway, acetyl-CoA carboxylase, was significantly up-regulated. This enzyme converts acetyl-CoA to malonyl-CoA, a key inhibitor of FAO. These results suggest that FAS is up-regulated and FAO is down-regulated in monocytes from kidney disease subjects. Similarly, the up-regulation of both transcription regulators SREBP and ChREBP supports an increased FAS enzyme transcription and activity in kidney disease subjects.

Carnitine transports fatty acids into mitochondria via CPT1, CPT2, while CAT transfers the results of FAO, acetyl group to carnitine to be transported out of mitochondria. CPT 1A was significantly down-regulated, while CPT 1B (muscle and heart isoform) and CPT 2 transcription did not change. In addition, there was no change in CACT. Malonyl CoA is a significant regulator of CPT1, whereas increased malonyl-CoA inhibits CPT1.

These data suggest that FAS and inhibition of FAO, and polarization of macrophages to the M1 typing, in CKD subjects contributes to an inflammatory environment more so than in Healthy controls. The data also support increased cell longevity, which does not conform to traditional macrophage typing. Longevity appears to be supported by FAO.[107]

Gene transcription of CKD monocytes supports an environment with increased monocyte proliferation, cell survivability and viability, and decreased cell death, apoptosis, and necrosis. Cholesterol is an important component of most membranes. It is an integral component of lipid rafts, which are necessary for signaling complexes. Cholesterol synthesis supports the anabolic needs of the highly proliferative state of the CKD monocyte environment compared to monocytes from Healthy controls.

Among the canonical pathways in our data, cholesterol synthesis is the most significantly up-regulated pathway for CKD subjects compared to controls (p-value: 0.000000002; z-score: 4.243). Likewise, the key regulatory enzyme in FAS is significantly up-regulated, (ACACA; p-value: 0.002). Over production of cholesterol and fatty acids can be toxic to cells. Control of these two biosynthetic pathways involves a feedback regulatory system that senses intracellular levels of cholesterol and fatty acids and alters transcription of genes encoding lipogenic enzymes. SREBP isoforms are transcription regulators that are membrane proteins located in the endoplasmic reticulum and modulate cholesterol and FAS. These membrane proteins have sterol response elements, which interact with genetic elements. While SREBP1a and SREBP1c are

more active in driving FAS, SREBP2 is more active in stimulating transcription of genes involved in cholesterol synthesis.[182]

Our data report that SREBF1 transcription is not changed relative to Healthy (p-value: 0.086); whereas SREBF2 transcription is significantly increased. p-value: 0.016). These results are consistent with the observation that FAS was minimally stimulated, primarily through ACACA with no transcriptional change in FASN enzyme complex. In contrast, enzymes of cholesterol synthesis were stimulated uniformly, including the key regulatory enzyme HMG-CoA reductase (p-value: 0.00003).

Accumulation of cellular cholesterol leads to activation of other transcription factors as well, including the liver X receptor α and β (LXRA, LXRB), the retinoid x receptor (RXR) and members of the peroxisome proliferator-activated receptor (PPAR) family including PPARA and PPARG.[107] Our data indicate PPAR, and LXR/RXR signaling pathways are significantly down-regulated in CKD compared to Healthy controls. Additionally, both individual genes, PPARA (p-value: 0.01) and PPARG (p-value: 0.004) transcription are down-regulated, while RXRA isoform is unchanged (p-value: 0.8).

Since one of the primary functions of LXRs is a cholesterol sensor, similar to SREBP, one of the best-known actions of LXRs is regulating reverse cholesterol transport. In macrophages, LXR reduces cellular cholesterol levels by up-regulating the expression of cholesterol transporters ABCA1 and ABCG1 and by up-regulating ApoE expression, which acts as a receptor for cholesterol. Mice with absent ApoE expression in macrophages are more susceptible to atherosclerosis. Increased production of lipoprotein remodeling proteins like LPL is consistent with LXRs function in macrophages to reduce cholesterol.[107]

In this study, transcription of LXR/RXR signaling pathway was down-regulated. Its importance in cholesterol efflux highlights the differences in CKD cholesterol accumulation. With lower transcription of LXR, there was a greater tendency for accelerated cholesterol accumulation in CKD subjects compared with Heathy controls.

One of the mechanisms for down-regulating cholesterol synthesis is through the internalization of lipoproteins through the LDL receptor (LDLR).[183] However, our data suggest that LDLR was up-regulated and yet SREBP2 transcription was increased. Others have reported

that with high cholesterol feeding and an over accumulation of cholesterol, there was both a failure of SREBP inhibition and processing and a significant elevation of HMG-CoA reductase. It was determined that a regulatory element of SREBP, INSIG (insulin induced gene) inhibits SREBP from moving to the nucleus and stimulates HMG-CoA reductase degradation.[182] Consistent with these results, our data support a significant increase in INSIG1 (p-value: 0.0001) and INSIG2 (p-value: 0.002) in CKD subjects. Use of statins may be another potential cause of elevated LDLR in CKD subjects, as statins are widely prescribed for CKD patients and result in increased production of HMGR and LDLR.[184]

A key atherogenic effect of cholesterol is through its oxidation. Oxidized LDL stimulates scavenger receptors, which leads to the internalization of oxidized lipoprotein particles with cholesterol via scavenger receptors instead of through the LDLR. Our data suggest that CKD patients have an increased expression of LDLR compared to Healthy controls (p-value: 0.00004), and that most of the scavenger receptors are either down-regulated or not changed. Lower levels of scavenger receptor transcription in CKD suggest that the level of oxidized lipoprotein is insufficient to up-regulate scavenger receptor transcription. This is not surprising given that the monocytes were incubated in serum for only 4 hours, and then in serum free media for 12 hours; under these conditions there may not have been sufficient time to generate oxidized lipoproteins. Cholesterol influx-efflux data, in conjunction with the elevated INSIG transcription, suggest that INSIG gene products may help regulate cholesterol synthesis even with the lack of responsiveness of SREBP2 to elevated cellular cholesterol levels, in part due to increased influx and decreased efflux.

The superpathway of cholesterol synthesis is composed of several canonical pathways: mevalonate pathway, superpathway of geranylgeranyldiphosphate synthesis I, cholesterol synthesis I, II, and III (data not shown for cholesterol synthesis II and III). In addition to the overall up-regulation of superpathway of cholesterol synthesis, all of these pathways separately were significantly up-regulated in CKD subjects compared to Healthy controls.

The mevalonate pathway and the superpathway of geranylgeranyldiphosphate (GGPP) biosynthesis I contain the initial steps of cholesterol synthesis; they are similar and overlapping. These pathways are anabolic and use acetyl-CoA derived from glucose, glutamine, and/or acetate metabolism to produce sterols and isoprenoid metabolites essential for a variety of biological processes. The mevalonate pathway, also known as HMG-CoA reductase pathway, produces two

5-carbon building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are used to make isoprenoids such as cholesterol, heme, vitamin K, coenzyme Q, and all steroid hormones. Mevalonate pathway stops with IPP, whereas superpathway of geranylgeranyldiphosphate continues three enzymes further in cholesterol synthesis to farnesyl diphosphate. The superpathway of geranylgeranyldiphosphate biosynthesis I diverges from cholesterol synthesis at farnesyl diphosphate (FPP) with the enzyme granyltranstransferase (gene coding: FDPS) which synthesizes GGPP. GGPP is used for protein isoprenylation, ubiquinone synthesis, or dolichol synthesis, which is used for N-glycosylation. Because the literature often does not differentiate between these pathways, they will be combined for purpose of this discussion and will collectively be referred to as the MVA pathway.[184,185]

Mevalonate kinase (MK) is the second essential enzyme of the cholesterol biosynthesis pathway and is subject to feedback inhibition by intermediates of cholesterol synthesis. Among CKD subjects in this study, MK transcription was significantly increased (p-value: 0.004).

The MVA pathway has been considered to be oncogenic, as many of its metabolites support growth and tumorigenesis. MVA is the only source of IPP, which when overproduced by cells causes T-cells to terminate the cell. Both FPP and GGPP support prenylation of several GTPases, such as Rho, Ras, Rac, and Rab, which play a role in cell growth, motility, intracellular signaling, and malignant transformation of cells. Inhibition of the MVA can reduce isoprenylation of these GTPases and induce cancer cell death in some cancer cells, which is reversed by addition of GGPP and FPP. There does not seem to be one or several GTPases responsible for cell survivability, but rather a class effect with the depletion of isoprenoid pools affecting many proteins that are isoprenylated.[185]

MVA produces quinone coenzyme Q as one of its metabolites. This isoprenoid localizes in the inner mitochondrial membrane where it transfers electrons from complex I or II to complex III of the electron transport chain, thereby enabling ATP production. Energy production capability is an essential component of proliferative cells.[185]

The RANK-NF-KB system plays a central role in several pathogenic developments in CVD and CKD including inflammation, apoptosis, and calcification. Transcription of NFKB1 and NFKB2 were significantly increased in CKD subjects (p-values: 0.002 and 0.004), presumably leading to a cascade of events. Our data did not support increased transcription of RANKL or

decreased transcription of OPG, which could result in increased signal transduction through RANK. Signaling can also be accomplished via decreasing the available decoy receptor, OPG, through its binding to TRAIL – gene coding: TNFSF10, and thereby reducing OPG inhibitory effect on RANKL-RANK binding. TRAIL was significantly increased in CKD subjects (p-value: 0.002). TRAIL is also a pro-apoptotic factor, which is in contrast to the developing picture of our monocyte/macrophage environment of CKD subjects. Binding of OPG to TRAIL decreases the inhibitory effect of OPG on RANKL activation of RANK; it also inhibits TRAIL's pro-apoptotic factor, facilitating an anti-apoptotic, pro-proliferative environment.[186,187]

RANK, similar to other TNF receptors, lacks a tyrosine kinase and therefore requires one to transmit cell signaling; often the tyrosine kinase is in the form of TNF-receptor associated factor (TRAF).[186] Our data show both TRAF1 (p-value: 0.0001) and TRAF3 (p-value: 0.000002) were up-regulated in CKD subjects. RANK/RANKL signaling is conducted by three pathways, Src/PLCγ , PI3K/Akt/mTOR, and MAPK (p38, JNK, ERK1,2) cascades which result in the translocation of transcriptional activators including NF-KB, Fos/Jun, or MITF, and leading to the transcription of several effector genes.[186] Our data support an up-regulation of Src and PI3K, but not Akt, and down-regulation of p38, but with activation of JNK.

RANK is not the only activator mechanism of NF-KB, but is part of the canonical pathway. CD40 activates NF-KB via NF-KB inducing kinase (NIK) and TRAF3 in the Non-canonical pathway. The third or Atypical activation of NF-KB occurs due to cellular stress such as hypoxia and reactive oxygen species (ROS) via casein kinase II (CK II; gene coding: CSNK2A1). Each pathway drives transcription of its own subset of inducible genes.[188]

TRAF1 and TRAF2 form a heterodimeric complex to activate MAPk8/JNK and NF-KB. The heterodimeric complex also interacts with inhibitors of apoptosis proteins (IAPs), BIRC2, BIRC3 thereby mediating the anti-apoptotic signals of TNF receptors.[189,190] Increased expression of TRAF1 has been reported to cause degradation of TRAF3, stabilization of NIK (non-canonical NF-KB activation pathway), and processing of p100 producing p52.[190] TRAF1is up-regulated by several stimuli and may modulate the interaction of TRAF2 with BIRC2 and BIRC3.[190]

TRAF3 participates in signal transduction via the non-canonical pathway of CD40, resulting in NF-KB activation and cell death, and inhibits TRAF2-mediated activation of NF-KB. It also

down-regulates proteolytic processing of NFKB2, thereby inhibiting its activation.[191,192] Our data suggest a significant up-regulation of CD40 as well (p-value: 0.0006).

Further, our data suggest that elements of canonical activation (RANK signaling and increase in TRAF1), non-canonical activation (up-regulation of CD40 and TRAF3), and atypical activation of NF-KB (up-regulation of CK II) occurred in CKD subjects compared to Healthy controls. Although these pathways may seem contradictory, they provide feedback regulation for each other. It is also clear that the canonical pathway of NF-KB activation stimulates its own feedback through increased transcription of inhibitors of its activation, e.g., inhibitor of NF-KB (IkBa or IkBb).[188] Our data support an up-regulation of IkBa in CKD subjects (p-value: 0.008).

Nuclear Regulatory Factor-2 (NRF2)-mediated oxidative stress response pathway was significantly up-regulated in CKD subjects (p-value: 0.00002, z-score: 3.7), and was second only to cholesterol synthesis in significance of up-regulation. NRF2 is a leucine zipper protein (gene coding: NFE2L2) that is part of a small family of transcription activators that bind to antioxidant response elements (ARE) in the promoter regions of target genes. This family of genes is important for the coordinated up-regulation of genes in response to oxidative stress, inflammation, and the regulation of cellular redox conditions. For example CCT7, CLPP, and FKBP5 were significantly up-regulated in CKD subjects. These genes code proteins that act as chaperone and stress response proteins that facilitate protein repair. GCLC, GCLM, CBR1, and AKR also were significantly up-regulated. These genes code for proteins that help in metabolism of xenobiotics and reactive metabolites, stimulating cell survival and tumorigenesis. SOD, TXN, GSR, and TRXR1 (and ATF4) are antioxidant proteins that facilitate a reduction in oxidative damage.

Interleukin (IL-8) signaling pathway was also significantly up-regulated in CKD subjects. IL-8 is a member of chemokines that plays a central role in angiogenesis, tumor growth, and inflammation. The cell surface receptors for IL-8 are coupled to G proteins. IL-8 also induces NF-KB through a TRAF6-dependent pathway, leading to the transcription of proangiogenic genes like ICAM and VCAM, an increased activation of VEGFR, and activation of Rho kinase, which promotes endothelial gap formation and enhances monocyte entrance into intimal space. IL-8 stimulates the up-regulation of matrix metalloproteinase (MMP2 and MMP9) expression, leading to increased endothelial cell migration. Migration and gap formation in endothelial cells lead to increased vascular permeability. Our data support an increase in ICAM, VEGFR, and

MMP9, but not Rho kinase in CKD subjects, supporting the increased binding of monocytes to endothelial cells and increased endothelial cell migration. Due to the stimulation of angiogenesis and cell migration, IL-8 is associated with stimulation of tumor growth and metastasis.

MMP9 was one of the most significantly up-regulated gene in the CKD subjects (p-value: 0.00000000003). It is involved in 2 significant canonical pathways in our data: IL-8 signaling pathway and neuroinflammation signaling pathway, Although over 20 MMPs have been identified, MMP9 is most closely associated with CKD.[31] MMPs functions as collagenases, geletinases, and other degradatory enzymes and are important regulators of extracellular matrix degradation and remodeling. Inflammation stimulates cytokines and growth factors associated with fibrosis.[137] The accumulation of fibrillar collagens leads to irreversible dysfunction and results in heart failure and end-stage renal failure. MMPs are traditionally believed to suppress fibrosis because of their proteolytic activity, MMPs, particularly MMP9, play key roles in stimulating fibrillar extracellular matrix (ECM) collagen accumulation.[31,140,141]

MMP9 is involved in more than regulating extracellular matrix; for example, in brain disorders, it appears to be involved in immune/inflammation responses that are related to the enzyme's ability to activate cytokines and chemokines as well as its ability to facilitate movement of monocytes into the extravascular brain tissue.[193]

MMP9 has been reported to exhibit similar mechanisms with cardiac and renal aging.[31] For example, oxidative stress induces MMP9 up-regulation. Levels of NO decrease MMP9.[31] Age-related increases in pro-inflammatory genes are attenuated with MMP9 gene deletion. MMP9 can affect macrophage polarization. The increase in M1/M2 proportion of macrophage observed with age in mice is blunted with MMP9 gene deletion.[31]

Both animal and human studies report events associated with increased arterial stiffness and prevalence of MMP2 and/or MMP9. Investigators showed that increased intimal and medial expression of MMP2 was associated elastin fragmentation in rats,[194] and localizes in areas of elastin fragmentation.[195] In humans, serum MMP2 and MMP9 levels and MMP9 genes were associated with increased arterial stiffness.[196] Tissue expression of MMP2 was reported to correlate with increased elastin fragmentation, PWV, and calcium deposits in renal transplant patients.[197]

Others have reported a significant negative relationship between PWV and serum MMP2 and MMP9 in Healthy subjects.[198] Structural changes of collagen fibers, in particular collagen cross-linking by advanced glycation endproducts (AGEs), may also affect arterial stiffness. For example, suppression of AGEs by aminoguanidine in animal models prevents arterial stiffening without alterations in collagen or elastin content. [199] In human studies, treatment of hypertensive patients with ALT-711, a non-enzymatic breaker of collagen cross-links, resulted in a significant reduction in PWV. [48,200]

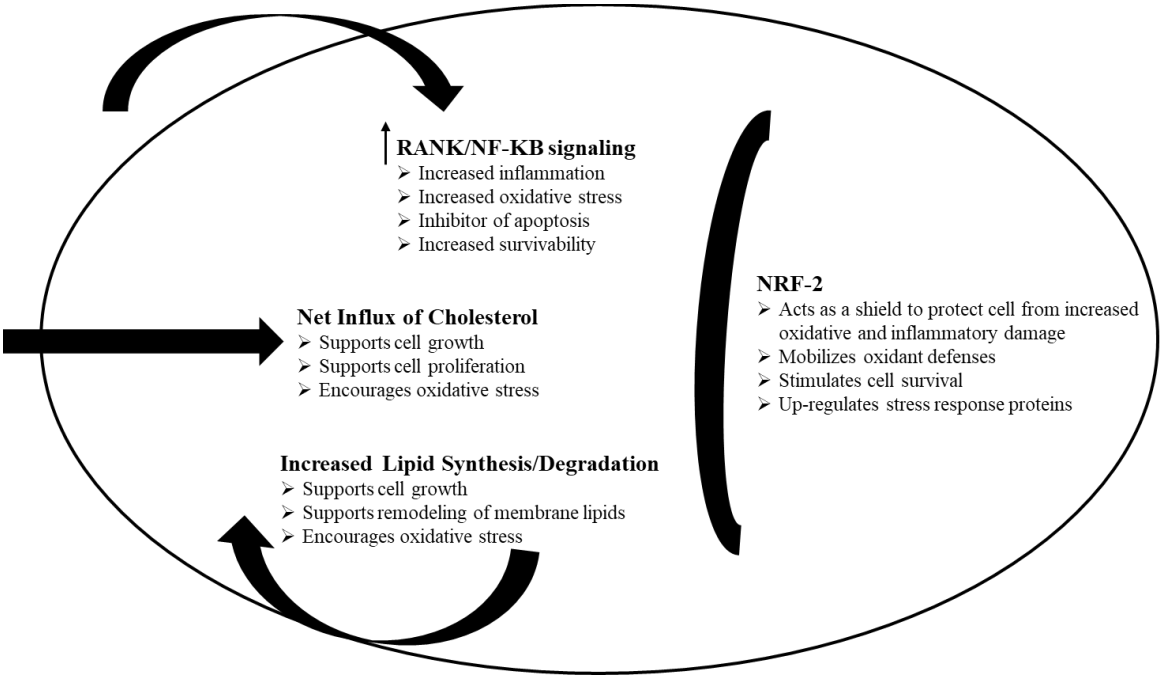
Production of NO and ROS in macrophages pathway are significantly up-regulated. There are 2 major production systems: production of NO by inducible NOS (iNOS), and production of ROS by NADPH oxidase on plasma membrane or phagosomal membrane. The former pathway was significantly up-regulated in CKD subjects (p-value: 0.02, z-score: 2.4) as was the iNOS signaling pathway (p-value: 0.009, z-score: 2.3). There were several enzymes with significant up-regulation in the production of NO, specifically in the activation cascade stimulating NF-KB transcriptional. iNOS is not transcriptional up-regulated. Transcriptional up-regulation of iNOS may not be indicative of NO generation, however, since there is significant post transcriptional regulation of iNOS mRNA, especially by tristetraprolin (TTP) [201] Our data suggest that TTP was significantly down-regulated in CKD (p-value: 0.003), suggesting a mechanism for increased iNOS availability with similar transcription levels. Further, calmodulin complex, which is sensitive to intracellular calcium, was significantly up-regulated. Jones et al. demonstrated that calmondulin-dependent protein kinase is required for proper localization and functioning of iNOS in vascular smooth muscle cells.[202] Regarding, ROS production, several enzymes synthesizing ROS were significantly unregulated in CKD subjects, including p47 phox, p67 phox, p22 phox, gp91, and in the process, Rho was significantly stimulated.

In this study TNFR1 signaling pathway was significantly up-regulated (p-value: 0.003, z-score: 2.1). As in RANK signaling, TRAF receptor was significantly up-regulated (p-value: 0.0001), along with two cIAP (cellular inhibitor of apoptosis), BIRC2 (p-value: 0.0002) and BIRC3 (p-value: 0.002). Signaling cascade involves NF-KB signaling similar to production of NO pathway, resulting in significant up-regulation of several enzymes in the cascade and translocation of NF-KB to the nucleus.

If gene transcription data translated directly to protein abundance and biological function, then the net effects of differential transcription for CKD subjects relative to Healthy controls

could be summarized in part in Figure 2. The central role of the RANK signaling and NF-KB activation in CKD monocytes is reflected below with their involvement in survivability, apoptosis, inflammation, and oxidative stress. Likewise, monocytes are up-regulating a net cholesterol influx that supports cell growth and proliferation and leads to increased oxidative stress. Likewise, increased lipid synthesis and degradation support cell growth and membrane remodeling, but also leading to increased oxidative stress. Finally, there is a significant, coordinated upregulation of monocyte oxidative protection mechanisms that help to lessen oxidative damage that occurs with increased metabolic turnover.

Figure 2: Summary of Differential Gene Transcription: Proposed Effects on Monocytes



The study was limited in that its interpretation was based only on transcription data, with no corroborating proteomics data. Further, there were no measures of biological function to corroborate our transcription data.

In humans, mRNA concentrations account for 30-40% of the variation in protein abundance. The other 60% of the variance is due to post-transcriptional regulation, translational regulation, and protein degradation.

In general protein abundance depends on a protein's biological role. Regulatory proteins are produced and degraded rapidly in response to a stimulus, whereas structural or house-keeping

proteins have longer half-lives. After contributions of mRNA abundances (i.e., transcription and mRNA degradation) to protein abundance variance are removed, the next highest contributors are translational regulation and protein degradation, with translation being the more significant contributor. It has been suggested that mRNA is a good indicator for the presence of a protein even though mRNA explains only a fraction of protein abundance.[203] Vogel and Marcotte suggested that regulation of transcript abundance could be thought of as controlling the on or off state of each gene and setting the order of magnitude of protein abundances. A combination of post-transcriptional, translational, and degradative regulation, acting through micro RNAs or other mechanisms, then fine-tunes protein abundances to their preferred levels.[204]

Overlaying these regulatory effects on protein abundance described above is the recently discovered small (20–30 nucleotide) noncoding RNAs that regulate genes and genomes. The regulation can include RNA processing, RNA stability, transcription, and translation. Three main categories of regulatory RNA have been identified: piwi-interacting RNAs (piRNAs), short interfering RNAs (siRNAs), and microRNAs (miRNAs).[205]

According to Vogel and Marcotte, there is surprisingly little variance contributed to protein abundance from biological or experimental noise.[204] Regardless, given that our sample size was 3 and that these data contained outliers, weighting of samples was necessary in order to improve data confidence. A weighted ANOVA model was used in conjunction with empirical Bayes adjusted contrasts to make comparisons (see Methods for discussion of benefits of this method).

Finally, our data are limited in that they were derived from circulating monocytes which were induced to become adherent monocytes. In contrast, monocytes contribute to atherosclerotic processes in large part through their transformation to macrophages.

The current work could be extended in a number of ways. Monocytes and their environment change with age as well as with degree of disease, and further studies could evaluate the effect of these variables on study results. Monocytes/macrophages exhibit more pro-inflammatory characteristics with age and with degree of atherosclerosis and calcification. This work could be extended by examining monocytes from CKD patients and matched Healthy controls at several levels of CKD, including endstage renal disease. In addition to examining monocytes, it may also be informative to evaluate the degree of calcification of central arteries and level of metabolites in the blood. Further, it would extend this work to examine the same parameters in

diabetic versus non-diabetic subjects. In addition, because the growth and cancer-like activity of the monocyte environment is fueled by energy, most likely carbohydrate, it is interesting to speculate what would happen if this energy source became less available.

In conclusion, monocytes from CKD subjects exhibited increased survivability, due in part to their reduced apoptotic signaling and increased membrane remodeling. These monocytes exhibited increased inflammatory signaling and oxidative stress compared with Healthy controls.

VII. Conclusions

Our data support increased influx and decreased efflux of cellular cholesterol, increased cholesterol synthesis, increased FAS, increased RANK/NF-KB signaling, increased inflammation and oxidation, and decreased FAO of the CKD incubated monocytes compared to Healthy matched control monocytes. Monocytes in the current study were incubated for a relatively short duration, considering that monocytes were incubated 5 days to transform into macrophages.[179] Accumulation of cholesterol and membrane lipids occurs in monocytes.[169] Differences in transcription between these groups could be due differences in monocytes in addition to the differences outlined above regarding CKD blood.

Monocytes are important contributors to inflammation, immune defense, clearing pathogens and dead cells, providing precursors pool for dendritic cells and macrophages, as well as tissue repair.[179] Their function, however, is dependent on monocyte subset and local environment.[179] Circulating LDL and Lp(a) interact with monocytes and contribute to an enhanced inflammatory state. For example, monocytes from patients with familial hypercholesterolemia have increased expression of CD11b, CD18, and chemokines receptors CX3CR1 and CCR2.[180] Increase in CCR2 expression is correlated with elevated lipid accumulation in the monocytes and helps mediate monocytes toward vascular sites of inflammation.[169,180] Similarly, monocytes isolated from patients with familial dysbetalipoproteinemia, characterized by elevated plasma triglycerides and cholesterol, are phenotypically inflamed with increased expression of CD11b/CD11c/CD18.[181] In fact, the altered composition of monocytes derived from hypercholesterolemic patients results in different behavior, as shown by ex vivo increased transmigration capacity across endothelium.[169,180]

As noted above, Figure 2 summarizes the biological effects in monocytes for CKD subjects relative to Healthy controls if differential gene transcription translated directly to biological endpoints. RANK signaling and NF-KB activation in CKD monocytes played a central role being involved in monocyte survivability, apoptosis, inflammation, and oxidative stress. Likewise, cholesterol influx is up-regulated in CKD monocytes supporting cell growth and proliferation and leading to increased oxidative stress. Likewise, increased lipid synthesis and degradation support cell growth and membrane remodeling, but also lead to increased oxidative stress. Finally, there is a significant, coordinated upregulation of monocyte oxidative protection mechanisms that help to lessen oxidative damage that occurs with increased metabolic turnover.

These data link increased monocyte internalized lipid and an increased inflammatory state in subjects with CKD. In our pilot study, fresh monocytes from CKD subjects exhibited increased inflammatory state compared to matched Healthy controls, as evidenced by increased expression of CCR2 and CX3CR1. After incubation, monocytes from CKD subjects exhibited enhanced inflammatory profile, as evidenced by increased expression of CD11b, CD11c, and CD18. Our data suggest that there was a relatively rapid increase in genes involved in cholesterol and lipid metabolism, as well as genes involved in inflammation and oxidation among subjects with CKD relative to Healthy controls.

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