Advanced glycation endproducts and inflammatory cytokine profiles in maintenance hemodialysis patients after the ingestion of a protein dense meal

Ryan K. Perkins, PhD, Stephan van Vliet, PhD, Edwin R. Miranda, MS, Kelly N.Z. Fuller, PhD, Paul J. Beisswenger, MD, Kenneth R. Wilund, PhD, Scott A. Paluska, MD, Nicholas A. Burd, PhD, Jacob M. Haus, PhD

PII: S1051-2276(21)00295-8
DOI: https://doi.org/10.1053/j.jrn.2021.11.006
Reference: YJREN 51815

To appear in: Journal of Renal Nutrition

Received Date: 16 August 2021
Revised Date: 11 November 2021
Accepted Date: 28 November 2021


This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2021 Published by Elsevier Inc. on behalf of the National Kidney Foundation, Inc.
Advanced glycation endproducts and inflammatory cytokine profiles in maintenance hemodialysis patients after the ingestion of a protein dense meal

Ryan K. Perkins PhD¹, Stephan van Vliet PhD², Edwin R. Miranda MS¹, Kelly N.Z. Fuller PhD⁵, Paul J. Beisswenger MD⁵, Kenneth R. Wilund PhD²,³, Scott A. Paluska MD⁴, Nicholas A. Burd PhD²,³, and Jacob M. Haus PhD¹

¹School of Kinesiology, University of Michigan, Ann Arbor, MI, ²Department of Kinesiology and Community Health, University of Illinois at Urbana-Champaign, Urbana, Illinois, ³Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois, ⁴Department of Family Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois, ⁵Kansas University Medical Center, ⁶Geisel School of Medicine, Dartmouth College, Lebanon, NH

Corresponding Author
Jacob M Haus, PhD
jmhaus@umich.edu
(734) 647-2790

Word Counts
Abstract – 273
Manuscript body – 4279

Short Title (Running Head)
Inflammatory milieu in MHD after a mixed meal

Acknowledgements
We would like to thank the subjects for graciously volunteering to participate in the study. We would also like to thank the Champaign-Urbana Dialysis Center for encouraging recruitment efforts.

Funding: This research was supported by a grant from the American EggBoard/Egg Nutrition Center and National Institutes of Health R01 DK109948. SvV was supported by a European Society for Clinical Nutrition and Metabolism Research Fellowship. Clinical Trial Registration: NCT03478722 (www.clinicaltrials.gov).

Conflicts of Interest
None declared.

Potential Reviewers
Biruete, Annabel abiruete@iu.edu
Kovesdy, Csaba Pal ckovesdy@uthsc.edu
Ikizler, Alp alp.ikizler@vumc.org
Slivka, Dustin dslivka@unomaha.edu
Advanced glycation endproducts and inflammatory cytokine profiles in maintenance hemodialysis patients after the ingestion of a protein dense meal

Word Counts
Abstract – 267
Manuscript body – 4272

Short Title (Running Head)
Inflammatory milieu in MHD after a mixed meal

Acknowledgements

Conflicts of Interest
None declared.
Abstract

Objective: The goal of this investigation was to evaluate circulating and skeletal muscle inflammatory biomarkers between MHD and demographic-matched control subjects (CON) before and after ingestion of a protein rich meal. Design and Methods: CON (n=8; 50±2 y; 31±1 kg/m²) and MHD patients (n=8; 56±5 y; 32±2 kg/m²) underwent a basal blood draw and muscle biopsy and serial blood draws after the ingestion of a mixed meal on a non-dialysis day. Plasma advanced glycation end products (AGEs) and markers of oxidation (OX) were assessed via LC-MS/MS before and after the meal (+240 min). Circulating inflammatory cytokines, soluble receptor for AGEs (sRAGE) isoforms (endogenous secretory RAGE, esRAGE; cleaved RAGE, cRAGE) were determined before and after the meal (+240 min). Basal muscle was probed for inflammatory cytokines and protein expression of related signaling components (RAGE, TLR4, OST48, TRIF, total and pIκBα). Results: Basal circulating AGES were 7-343 fold higher (P<0.001) in MHD than CON but only MG-H1 increased in CON following the meal (P<0.001). There was a group effect (MHD>CON) for total sRAGE (P=0.02) and esRAGE (P<0.001) and a trend for cRAGE (P=0.09), with no meal effect. Additionally, there was a group effect (MHD<CON; P<0.05) for circulating Fractalkine, IL10, IL17A, and IL1β and a trend (P<0.10) for IL6 and MIP1α, whereas TNFα was higher in MHD (P<0.001). In muscle, TLR4 (P=0.03), TRIF (P=0.002), and OST48 (P=0.02) expression was lower in MHD than CON, while IL6 was higher (P=0.01) and IL8 (P=0.08) tended to be higher in MHD. Conclusion: Overall, MHD exhibited an exaggerated circulating and skeletal muscle inflammatory biomarker environment and the meal did not appreciably affect inflammatory status.

Key Words
Maintenance Hemodialysis, Inflammation, AGEs, Diet
Introduction

The loss of muscle mass in patients undergoing MHD is well documented and reported to be a primary consequence of a pro-inflammatory state (1-3). Chronically elevated circulating advanced glycation endproducts (AGEs) are a major driver of inflammation in chronic kidney disease (CKD) patients (4-8) and progression along the CKD continuum is related to increased circulating AGE load (8, 9). AGEs are a heterogeneous group of molecules formed from the non-enzymatic reactions between glucose, or reactive α-dicarbonyls such as methylglyoxal, and positively charged amino acid residues (10-12). AGEs encourage pro-inflammatory events through recognition by the receptor for AGEs (RAGE) and subsequent downstream signaling pathway activation (e.g., NFκB) (7, 13-16). This ligand-receptor system can be disrupted through proteolytic release of RAGE, forming a cleaved RAGE isoform (cRAGE) (17) and alternative RAGE splicing, leading to the production and cellular expulsion of endogenous secretory RAGE (esRAGE) (18). cRAGE and esRAGE (total sRAGE) suppress AGE-RAGE signaling by: 1) reducing signaling potential at the cell due to fewer RAGE receptors; and 2) acting as a decoy through binding and sequestering circulating AGEs (7).

Inflammation disrupts protein homeostasis in maintenance hemodialysis (MHD), promoting a catabolic environment (19-21). In support of this link between inflammation and proteostasis in MHD, past efforts have shown a relationship between release of the pro-inflammatory cytokine interleukin-6 (IL6) from peripheral tissues and accelerated muscle protein loss (21). Furthermore, markers of circulating inflammation, such as CRP, are strongly related to indirect measurements of skeletal muscle protein synthesis, degradation, and net protein balance in MHD patients (19).

Proinflammatory cytokines exert their effects on protein balance by activating downstream proteolytic signaling pathways, including the ubiquitin-proteasome pathway and caspase-3. We recently demonstrated individuals undergoing MHD have higher basal caspase-3 protein content in skeletal muscle (22). Activation of pathways that regulate protein breakdown rates leads to the excess release of amino acids into the muscle intracellular free pool resulting in elevated muscle protein synthesis.
rates in the basal-state (23). This overstimulation of MHD muscle results in anabolic resistance of muscle protein synthesis rates to dietary protein and over time likely contributes to accelerated losses in muscle mass in individuals on MHD (23).

Moreover, protein energy wasting (i.e., depletion of body protein stores) is another hallmark of advanced CKD and likely contributes to the progression of muscle loss with MHD. Therefore, it is not surprising that increasing the protein density of dietary patterns have received much attention due to the impact of renal replacement therapy on appetite and nutrient availability (20, 24-27). Evidence exists showing MHD treatment is catabolic in nature (28), an outcome potentially driven by modulation of genes involved with apoptosis and inflammation in skeletal muscle (29) and loss of amino acids in the dialysate (30). Coupled with the upregulation of inflammatory signaling pathways in muscle, loss of amino acids during MHD highlights the importance of consuming protein-rich meals between dialysis treatments to counteract the catabolic nature of MHD.

Despite continued illumination of the underlying mechanisms promoting inflammation, our understanding of the inflammatory phenotype in MHD is still limited. It is clear that patients on MHD experience muscle wasting, a condition suspected to be perpetuated by chronic basal inflammation and poor nutrient status. A more complete understanding of the inflammatory burden in patients undergoing MHD will direct targeted therapeutics. Thus, the goal of this investigation was to evaluate the spatial and temporal dynamics of inflammation and inflammatory regulators by evaluating a host of biomarkers in the circulation and skeletal muscle in the basal state and following a mixed, protein-dense meal recommended by the National Kidney Foundation for an MHD population (31) and a demographic-matched control group. Furthermore, we aimed to build on our previous work and explore a potential relationship between the inflammatory milieu and anabolic resistance to feeding observed in this MHD cohort (22).
Methods

Experimental design

Recruitment methods for this pilot study have been previously reported representing sub-analysis of this work (22). Briefly, volunteers were determined to be eligible based on responses to a detailed medical screening questionnaire and blood panel findings. Additionally, MHD participants received clearance to participate in the study from their personal nephrologist. All participants were informed about the experimental procedures and potential risks before providing written consent. The study was approved by the Institutional Review Board at ----- and conformed to standards for the use of human participants in research as outlined in the Declaration of Helsinki. Clinical Trial Registration: (www.clinicaltrials.gov).

Participants

MHD (n=8; 56±5 y) and CON (n=8; 50±2 y) participants were matched for age, sex, BMI, and HOMA-IR (Table 1). MHD participants were taking the following medications: phosphatase binders (n=8), calcimimetics (n=8), angiotensin-converting enzyme inhibitors (n=6), nonsteroidal anti-inflammatory drugs (n=6), β-blockers (n=4), calcium channel blockers (n=4), diuretics (n=4), statins (n=4), proton pump inhibitors (n=3), xanthine oxidase inhibitors (n=3), opioids (n=3), vasodilators (n=2), P2Y12 inhibitors (n=2), serotonin-reuptake inhibitors (n=2), antihistamine (n=1), prokinetic (n=1), and immunosuppressant (n=1). CON participants were taking nonsteroidal anti-inflammatory drugs (n=2). Body weight and height was assessed by standard procedures as well as body composition via dual-energy X-ray absorptiometry (DEXA) scan.

Protocol

A detailed description of the protocol has been described elsewhere (22). Briefly, all participants were instructed to refrain from vigorous physical activity for 3 days before the trial and maintain their
normal dietary pattern and prescribed medications. Subjects reported to the laboratory at 7:00 AM after consuming a 320 kCal standardized meal (22 g protein, 43 g carbohydrate, and 7 g fat) the night before. Other than this standardized meal, participants were instructed to remain fasted. MHD patients were studied ~24 h after their previous dialysis treatment. The study protocol consisted of a basal blood draw and skeletal muscle biopsy followed by serial blood draws (30, 60, 90, 120, 180, 240, and 300 min) after a mixed meal challenge. Circulating post-meal inflammatory assessments were made at the 240 min time point. Coupled with sample availability, our intention was to capture the inflammatory load in both the postabsorptive and postprandial states. Additionally, all participants performed a 2-day dietary recall (Nutritionist Pro v2.1.13, Axxya Systems, Redmond, WA). On average, protein intake tended to be lower ($p<0.10$) in MHD than CON (MHD: 0.9±0.14; CON: 1.14±0.05 g/kg BW). No differences in other macronutrients were observed ($p>0.05$).

**Mixed meal challenge**

After basal measurements, each participant consumed a mixed meal consisting of 3 scrambled eggs, 1 slice of toasted white bread, 300 mL apple juice, and 10 g cow butter (total: 546 kcal, 20 g protein, 59 g carbohydrate, 26 g fat). This meal was selected because it is representative of a standard meal recommended by National Kidney Foundation for this patient population (31). Macronutrient composition of the eggs was determined by the combustion method (method 990.03; AOAC International, 2000; TruMac; LEO Corp., Sain Joseph, MI). For the other foods, macronutrient composition was determined from their respective food label.

**Blood draws and skeletal muscle biopsy**

A catheter was inserted into a dorsal hand vein and heated for sampling of arterialized venous blood. A skeletal muscle biopsy (32-35) of the *m.* vastus lateralis was obtained in the basal state under local anesthetic (Lidocaine, 2%). Following the muscle biopsy, excess blood, visible fat, and connective
tissue was removed and the sample was immediately flash-frozen in liquid nitrogen and stored at -80 °C until analysis.

**Blood analyses**

Metabolites (glucose, albumin, creatinine, eGFR) were assessed using a point-of-care chemistry analyzer (Piccolo Xpress Chemistry Analyzer; Abaxis, Union City, CA). Basal plasma insulin and high-sensitivity C-reactive protein concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA; Alpco Diagnostics; Salem, NH) and nonesterified fatty acids (NEFA; basal, 30, 60, 90, 120, 180, 240, and 300 min post meal) were measured via enzymatic assay (Wako Diagnostics, Richmond, VA). Total plasma sRAGE (basal and 240 min post meal) was measured with an ELISA (DRG00, R&D Systems, Minneapolis, MN, USA). This total sRAGE quantification approach captures the cleaved (cRAGE) and endogenous secretory (esRAGE) isoforms. To quantify plasma esRAGE (basal and 240 min post meal), another ELISA was performed (K1009-1AS, One International, Mountain View, CA). Plasma cRAGE was determined by subtracting esRAGE from [total sRAGE (36-40). Plasma inflammatory targets (Fractalkine, IFNγ, IL10, IL17A, IL1β, IL6, IL8, MIP1α, and TNFα) were assessed via Milliplex MAP Human High Sensitivity magnetic bead panel (HSTCMAG-28SK, Millipore Corporation, Billerica, MA) in the basal state and 240 min after the meal challenge. Antibody beads, controls, buffers, serum matrix, and standards were prepared and the kit was run per manufactures guidelines.

Markers of protein oxidation (OX) (methionine sulfoxide, MetSO, and amino adipic acid, AAA) and AGE-free adducts (Nε-carboxymethyllysine, CML; Nε-carboxyethyllysine, CEL; 3-deoxyglucosone hydroimidazolone, 3DG-H; glyoxal hydroimidazolone-1, GH-1; and methylglyoxal hydroimidazolone-1, MG-H1) were measured in plasma samples obtained in the basal state and 240 min after the mixed-meal by liquid chromatography–tandem mass spectrometry (LC–MS/MS) as previously described.
Briefly, plasma ultrafiltrate (10 kDa cutoff filter; Amicon (Millipore; Burlington, MA) were separated with a mobile phase gradient of methanol and water containing 0.20% heptafluorobutyric acid and quantified in a blinded fashion using stable internal isotope standards as described previously (38, 40, 41).

**Skeletal muscle analyses**

RAGE, TLR4, TRIF, total IκBα, phosphorylated IκBα (pIκBα), and OST48 were measured by Western blot as previously described (38). Approximately 10-15 mg (wet weight) of frozen muscle tissue was homogenized by ceramic beads (lysing matrix D, FastPrep-24 homogenizer, MP Biomedical, Santa Ana, CA, USA) in 20 volumes of ice-cold buffer made with 150 nM NaCl, 1 mM Na₂ EDTA, 1 mM EGTA, 2.5 mM NA pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1% Triton, and 1 μg·ml⁻¹ leupeptin (Cell Signaling Technology, Beverly, MA) with an added 1X protease and phosphatase inhibitor cocktail (5872S, Cell Signaling Technology). Total protein concentration was determined via BCA assay (Pierce Biotechnology, Rockford, IL). Equal protein was loaded on a gradient gel (BioRad, Hercules, CA) and resolved using SDS-PAGE, transferred to a nitrocellulose membrane, and blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) in TBS for 1 h at room temperature. RAGE (1:500, ab3611, Abcam, Cambridge, MA), TLR4 (1:2000, sc293072, Santa Cruz, Santa Cruz, CA), TRIF (1:1000, ab180689, Abcam), pIκBα (1:1000, 9246, Cell Signaling), total IκBα (1:1000, 9242, Cell Signaling), OST48 (1:1000, sc74408, Santa Cruz) primary antibody incubations took place overnight, rocking, at 4 °C. Secondary antibody (TLR4: 926-68072; TRIF, RAGE, and IκBα: 925-68071; OST48 and pIκBα: 926-32212; 1:20000, LICOR Biosciences) incubations occurred for 1 h at room temperature while rocking. Protein expression (RAGE, TLR4, TRIF, and OST48) was visualized with a NIR system (LICOR Biosciences), normalized to total protein (Ponceau, Sigma Aldrich, St. Louis, MO), and quantified using Image Studio (LICOR Biosciences). Total IκBα and pIκBα were also visualized with the NIR system and quantified using Image Studio, but pIκBα was normalized to total IκBα. RAGE was detected as two distinct bands (native: 43 kDa;
glycosylated: 48 kDa). Expression of both bands was summed to represent total RAGE expression (and normalized to total protein) (38). While postprandial samples were collected in the study (Cite van Vliet Paper), due to tissue collection limitations, muscle protein targets were only assessed in the basal state.

Muscle inflammatory targets (Fractalkine, IFNγ, IL10, IL17A, IL1β, IL6, IL8, MIP1α, and TNFα) were determined by Milliplex magnetic bead assay as described above. Skeletal muscle samples were homogenized by ceramic beads (lysing matrix D; MP Biomedical; Irvine, CA) in 20 volumes of ice-cold PBS supplemented with 1X protease and phosphatase inhibitor (5872S, Cell Signaling Technology). Following bead homogenization, samples were sonicated 2x30 sec at 50% power on ice. Sonicated samples were then spun at 20000g for 30 min at 4°C and the supernatant was retained for total protein concentration assessment via BCA assay (Pierce Biotechnology, Rockford, IL) and cytokine analyses. Antibody beads, controls, buffers, matrix (prepared in the muscle homogenization buffer), and standards were prepared and the kit was run per manufactures guidelines.

**Statistical analyses**

Data were analyzed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and evaluated for normality with a Shapiro-Wilk test. Non-normally distributed data were log transformed before statistical analyses. A two-way analysis of variance (ANOVA) with repeated measures was used to compare circulating OX markers, AGEs, inflammatory factors, and sRAGE concentrations between MHD and CON and time points (basal and 240 min post-meal). A Tukey’s post hoc test was utilized to examine specific differences when appropriate. The Student’s t-test was used to compare subject characteristics, changes (absolute and percent) in circulating inflammatory concentrations, and basal inflammatory muscle protein expression between groups. Relationships between variables of interest
were analyzed via Spearman’s rho correlation. Significance was set at $P < 0.05$ and a trend toward significance was recognized as $P < 0.10$. Data are presented as mean±SE.

**Results**

*Subject characteristics*

Subject characteristics are presented in Table 1 and have been previously reported (22). These data are presented here for context to the study’s main objective. MHD and CON participants were matched for age, sex, BMI, and HOMA-IR and were therefore similar ($P > 0.05$). MHD underwent a dialysis period of 5±1 y. Additionally, 38% of MHD (>5 y) and 13% of CON (<5 y) participants reported a history of smoking. MHD exhibited higher circulating creatinine (+148%; $P < 0.001$), BUN (+109%; $P < 0.001$) and CRP (+85%; $P = 0.01$) but lower albumin (-8%; $P = 0.04$) and eGFR (-160%; $P < 0.001$). There were no differences ($P > 0.05$) in basal NEFA concentrations (MHD: 0.64±0.11; CON: 0.57±0.06 mM) or AUC (MHD: 97.5±14.3; CON: 100.1±8.8 AU) from baseline to 300 min post meal between groups.

*Circulating markers of protein oxidation (OX) and advanced glycation endproducts (AGEs)*

Plasma protein OX and AGEs in the basal and postprandial state are presented in Figure 1. Analysis of plasma OX yielded no basal differences ($P > 0.05$) in MethSO or AAA between MHD and CON. An effect of time was observed for MethSO ($P = 0.03$) and AAA ($P < 0.001$). For the circulating AGEs, basal concentrations were higher ($P < 0.001$) in MHD than CON (range = 7 to 343-fold greater). Following the meal, CEL, CML, 3DG-H and G-H1 remained unchanged ($P > 0.05$) in both groups, whereas MG-H1 increased 11.1-fold ($P < 0.001$) at 240 min in CON. While MG-H1 increased in CON, concentrations remained ~28 fold lower than MHD at 240 min of the postprandial period.
Circulating markers of inflammation

Basal total sRAGE (-32%), esRAGE (-53%), and cRAGE (-24%) were not statistically different \( (P > 0.05) \) between CON and MHD (Table 2). Following the mixed meal, total sRAGE, esRAGE, and cRAGE remained unchanged \( (P > 0.05) \). However, independent of time, there was an effect of group for total sRAGE \( (P = 0.02) \) and esRAGE \( (P < 0.001) \) and a trend for cRAGE \( (P = 0.09) \) to be higher in MHD than CON.

Basal cytokine concentrations were similar \( (P > 0.05) \) between MHD and CON (Figure 2). Following the mixed meal, all plasma inflammatory factor concentrations remained unchanged \( (P > 0.05) \). However, at both time points together (basal and 240 min post meal), there was a group effect \( \text{MHD} < \text{CON} \) for Fractalkine \( (P = 0.003) \), IL10 \( (P = 0.007) \), IL17A \( (P = 0.04) \), and IL1\( \beta \) \( (P < 0.001) \) and a trend for IL6 \( (P = 0.06) \) and MIP1\( \alpha \) \( (P = 0.07) \). Additionally, there was an effect of group \( \text{MHD} > \text{CON} \); \( P < 0.001 \) for TNF\( \alpha \).

Muscle protein expression

Basal receptor signaling component protein expression in skeletal muscle of MHD and CON participants determined via Western blot are presented in Figure 3. Contrary to our original hypotheses, TLR4 \(-27\%; P = 0.03\) , TRIF \(-74\%; P = 0.002\) , and OST48 \(-55\%; P = 0.02\) expression was lower in MHD than CON, while RAGE and pIkBo/total IkB\( \alpha \) was similar between groups \( (P > 0.05) \). To complement the inflammatory receptor protein expression, a targeted panel of nine inflammatory cytokines were also evaluated (Figure 4). Findings (pg target protein) were normalized three ways: pg/mg muscle wet wt (Figure 4), pg/mL sample homogenate analyzed, and pg/\( \mu \)g total protein. Subtle statistical differences were observed among the three normalization approaches (see supplemental Table 1 for pg/mL and pg/\( \mu \)g protein normalizations). When cytokine protein was normalized to mg muscle wet weight, basal IL6 \(+141\%; P = 0.01\) and IL8 \(+70\%; P = 0.08\) were higher in MHD than CON, whereas the other cytokines were similar \( (P > 0.05) \).
Relationships among variables of interest

To explore potential relationships between variables we conducted correlation analyses between clinical characteristics and biomarkers. Of note, for all participants in the basal state, we found an inverse relationship between body weight and plasma sRAGE \( (r = -0.52; P = 0.04) \) and esRAGE \( (r = -0.50; P = 0.05) \). As anticipated, basal plasma AGEs were strongly correlated with each other \( (r = 0.81-0.92; P < 0.001) \). Several basal plasma AGEs were inversely correlated with plasma IL10 \( (CML: r = -0.51, 3DG-H: r = -0.54, MG-H1: r = -0.55; P < 0.05) \), positively correlated with plasma TNF\(\alpha\) \( (CML: r = 0.86, 3DG-H: r = 0.80, CEL: r = 0.81, G-H1: r = 0.84, MG-H1: r = 0.70; P < 0.001) \) and muscle pg/\(\mu g\) protein IL6 \( (CML: r = 0.60, 3DG-H: r = 0.65, CEL: r = 0.64, G-H1: r = 0.69, MG-H1: 0.66; P < 0.05) \). Basal plasma AGEs were also inversely correlated with muscle TRIF expression \( (r = -0.55-0.78; P < 0.05) \). Overall, few relationships were observed in response to the mixed meal challenge. Interestingly, when correlated with basal muscle myofibrillar synthesis rate (FSR; as reported in our previous publication) \( (22) \), FSR was related to the percent change in plasma Fractalkine \( (r = 0.56; P = 0.03) \), IL10 \( (r = 0.52; P = 0.04) \), IL1\(\beta\) \( (r = 0.55; P = 0.03) \), and TNF\(\alpha\) \( (r = 0.53; P = 0.04) \).

Discussion

The overarching goal of this investigation was to provide insight into the inflammatory phenotype in MHD by evaluating a comprehensive, yet targeted group of circulating and skeletal muscle inflammatory biomarkers in the basal state and after a typical meal rich in protein recommended for this population. The principal findings from this study include: 1) circulating AGEs are substantially elevated in MHD patients compared to CON, 2) the basal circulating and skeletal muscle cytokine profile in MHD is generally pro-inflammatory, and 3) there was minimal effect of meal ingestion on modulating all inflammatory biomarkers in both groups.

A major objective of hemodialysis is to remove uremic solutes that exert toxic effects. Using the gold-standard quantification methods \( (42) \), we report that basal plasma AGE concentrations are 7
to 343-fold higher in the MHD patients when compared to demographic controls. Noteworthy is that these assessments were made on samples from a non-dialysis day (~ 24h after finishing dialysis), which suggests chronic AGE toxicity in MHD patients rather than only acutely during the dialysis period. Our results are consistent with previous reports (4, 43, 44), and note that MG-H1 is the most abundant AGE in MHD patients. Additional reports have observed a reduction in circulating AGEs in response to hemodialysis up to 86%, however circulating AGE burden remains substantially elevated compared to control subjects (43-45). Interestingly, the lack of correlation between reduction in AGE levels and AGE molecular size with dialysis (43) highlights factors other than filter pore size govern AGE removal, thus, modified filtration approaches may need to be reconsidered.

AGEs exert many of their effects by activating RAGE signaling (7, 13-16). Interestingly, RAGE expression was similar between groups and OST48, TLR4, and TRIF expression was lower in MHD than the control group. This is intriguing and to the best of our knowledge, the first report of RAGE protein expression in skeletal muscle of individuals undergoing MHD. In contrast to findings presented here, one of the few studies conducted on circulating human cells demonstrated elevated RAGE protein expression in peripheral blood monocytes (PBMCs) of dialysis patients (46) and another reported higher PBMC RAGE mRNA expression in advanced CKD patients (47). Findings of high RAGE expression in circulating cells and similar RAGE expression in patients with renal disease suggests a potential tissue-specific effect. Interestingly, OST48 expression was lower in MHD. OST48 is a membrane bound receptor that binds and transports AGEs from the extra- to intracellular compartments for processing and removal (48, 49). Therefore, OST48 may compete with RAGE for ligands and likely exerts overall anti-inflammatory effects. In support, we observed a strong relationship between muscle OST48 and muscle IL10 (r = 0.59; P = 0.02). MHD muscle containing less OST48 than control subjects may be a fundamental component of the generally pro-inflammatory environment.

TLR4 is tasked with recognizing a host of ligands (some of which are shared with RAGE) and signal to downstream pathway elements, including the TRIF adaptor and the NFkB pathway. Similar
to our work presented here in muscle, Kuroki et al. found reduced TLR4 expression in circulating cells compared to controls, and dialysis duration (in years) was inversely related to TLR4 expression (50). Other studies have shown no difference (51) or greater TLR4 expression in circulating cells of dialysis patients (52). To our knowledge, the only other study to examine TLR4 in muscle of this patient population found higher TLR4 expression in dialysis patients than a control group (53). The differential findings between our study and those by Verzola et al. may be explained by the analytical approach used to measure TLR4 (Western blot in our study vs immunohistochemistry by Verzola et al.) or the type of dialysis technique utilized by the patients (i.e., MHD in our study and peritoneal dialysis by Verzola et al.). This is important to consider as hemodialysis is inflammatory in nature and has been shown to modulate genes involved with apoptosis and inflammation in skeletal muscle (29).

sRAGE is antagonistic to RAGE, exerting its effects by lowering inflammatory burden and improving indices of clinical health (7, 18, 37, 54-56). Chronic sRAGE treatment in db/db mice reduces glomerulosclerosis and improves renal function (54). In humans, sRAGE is strongly associated with muscle mass (56), low sRAGE is related to the development of obesity and insulin resistance across the glucose tolerance continuum (37), and weight-loss is related to increased sRAGE (57). In line with other studies on patients undergoing dialysis (55, 58), here we report elevated total sRAGE and esRAGE in MHD patients. One explanation for higher sRAGE in our MHD patients is the reliance on the kidney for sRAGE clearance, as total sRAGE concentration decreases after kidney transplantation (59). Another potential explanation for exaggerated sRAGE levels in clinical patients, is this serves as a protective countermeasure to chronic inflammation. Therefore, in patients characterized by a pro-inflammatory environment, high sRAGE may be indicative of underlying pathologies. In fact, very high sRAGE is associated with cardiovascular disease and all-cause mortality in clinical populations (60). It is clear that sRAGE has protective effects, however, it remains to be seen if sRAGE is upregulated as a compensatory mechanism to combat the inflammatory load in MHD or if impaired renal function blunts sRAGE clearance.
Patients undergoing dialysis exhibit a pro-inflammatory phenotype, reflective of a dynamic balance that has shifted away from factors that resolve (e.g., IL10) towards factors that perpetuate (e.g., TNFα) inflammation (1-3, 20, 21, 52, 53). Overall, here we show the basal profile in MHD is pro-inflammatory. In the circulation, MHD had more pro-inflammatory CRP and TNFα and less anti-inflammatory IL10 than the control group. CRP is a well-studied inflammatory biomarker and is linked to negative forearm protein balance (19), often used as proxy for skeletal muscle, and muscle loss (61) in dialysis patients. CRP has also been shown to be localized to atherosclerotic lesions in humans and CRP treatment of human monocytes leads to TNFα production (62). Interestingly, we found a strong correlation between circulating CRP and TNFα concentration (r = 0.70; P = 0.004). TNFα drives muscle wasting through its activation of the nuclear-factor-kappa B (NFκB) pathway (63) and caspase 3 (64). Greater TNFα concentration in MHD muscle may explain the higher caspase 3 expression and hyper-stimulation of myofibrillar protein synthesis rates in MHD reported in our previous publication (22).

Provided muscle is a predominant metabolic tissue that produces and secretes inflammatory factors (65) and experiences substantial wasting in renal failure (66), it is surprising so little is known about inflammation concentrations in MHD muscle. In this study, we report a modest pro-inflammatory profile in MHD muscle as concentrations of the pro-inflammatory IL6 and chemotactic protein IL8, are elevated compared to the CON group. IL6 modulates its inflammatory effects in muscle through protein balance disruption. In support, short-term IL6 infusion into healthy humans reduces muscle protein turnover by 50% (67) and IL6 injection into animals is reported to induce muscle wasting and this effect is mediated by the suppressor of cytokine signaling 3 (SOCS3) (68). SOCS3 is generally considered proteolytic as it upregulates caspase 3. Therefore, elevated IL6 in MHD muscle may provide additional insight into greater caspase 3 protein content previously reported in this MHD cohort (22).

In summary, this study provides a comprehensive, yet targeted analyses of circulating and skeletal muscle inflammatory biomarkers in MHD patients in the basal state and after the ingestion of
a meal rich in protein. Our findings of higher AGEs and pro-inflammatory cytokines and lower OST48 and anti-inflammatory cytokines add to the growing literature indicating an overall pro-inflammatory environment in patients undergoing chronic MHD. Given the impact of renal replacement therapy on appetite and nutrient availability (20, 24-27), we also sought to explore the biomarker effect of a mixed meal. In general, there was minimal effect of the meal on biomarkers explored, indicating a typical meal for this population does not exacerbate or attenuate the pre-existing inflammatory load. These findings suggest that the overall patient care may need to evolve to generate more specific and complementary therapeutic approaches in CKD patients. The overarching goal should emphasize strategies that reduce inflammatory load, including tailored physical activity and nutritional programs, thereby alleviating anabolic resistance.

**Practical Applications**

In this study, we report an exaggerated circulating and skeletal muscle inflammatory environment, and a protein-rich meal did not appreciably affect inflammatory status. To curtail the loss in muscle mass MHD patients typically experience, these findings suggest dialysis treatments may need to evolve.


57. Miranda ER, Fuller KNZ, Perkins RK, Kroeger CM, Trepanowski JF, Varady KA, et al. Endogenous secretory RAGE increases with improvements in body composition and is associated with


Table 1. Clinical characteristics of control subjects (CON) and maintenance hemodialysis patients (MHD).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CON</th>
<th>MHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/F</td>
<td>6/2</td>
<td>6/2</td>
</tr>
<tr>
<td>Age (y)</td>
<td>50 ± 2</td>
<td>56 ± 5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>94 ± 4</td>
<td>94 ± 9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.7 ± 1.3</td>
<td>32.3 ± 2.4</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>29.4 ± 2.3</td>
<td>30.7 ± 3.5</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.7 ± 0.6</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>BUN</td>
<td>15 ± 1</td>
<td>32 ± 3*</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.9 ± 0.1</td>
<td>3.6 ± 0.1*</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.1 ± 0.1</td>
<td>7.4 ± 0.9 *</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.6 ± 4.2</td>
<td>11.9 ± 3.0*</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>82 ± 6</td>
<td>9 ± 1 *</td>
</tr>
</tbody>
</table>

Data are mean ± SE. BMI: body mass index; HOMA-IR, homeostatic model of assessment for insulin resistance; BUN, blood urea nitrogen; CRP, c-reactive protein; eGFR, estimated glomerular filtration rate; * P < 0.05 vs CON.
Table 2. Plasma total sRAGE and sRAGE isoforms in control subjects (CON) and maintenance hemodialysis patients (MHD) in the basal state and 240 min after a mixed meal.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>CON (n=8)</th>
<th>MHD (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal (pg/mL)</td>
<td>240 min (pg/mL)</td>
</tr>
<tr>
<td>Total sRAGE *</td>
<td>932 ± 122</td>
<td>878 ± 120</td>
</tr>
<tr>
<td>esRAGE *</td>
<td>244 ± 37</td>
<td>239 ± 39</td>
</tr>
<tr>
<td>cRAGE †</td>
<td>688 ± 92</td>
<td>639 ± 86</td>
</tr>
</tbody>
</table>

Data are mean ± SE. sRAGE, soluble receptor for advanced glycation end products; esRAGE, endogenous secretory receptor for advanced glycation end products; cRAGE, cleaved receptor for advanced glycation end products; * Group effect: P < 0.05; † Group effect: P < 0.10.
Figure 1

- **A**: MethSO
  - Basal: CON (3000 nM) vs. MHD (1500 nM)
  - 240 min: CON (1500 nM) vs. MHD (3000 nM)

- **B**: AAA
  - Basal: CON (2100 nM) vs. MHD (1050 nM)
  - 240 min: CON (1050 nM) vs. MHD (2100 nM)

- **C**: CEL
  - Basal: CON (1200 nM) vs. MHD (600 nM)
  - 240 min: CON (600 nM) vs. MHD (1200 nM)

- **D**: CML
  - Basal: CON (1900 nM) vs. MHD (950 nM)
  - 240 min: CON (950 nM) vs. MHD (1900 nM)

- **E**: 3DG-H
  - Basal: CON (3300 nM) vs. MHD (1650 nM)
  - 240 min: CON (1650 nM) vs. MHD (3300 nM)

- **F**: G-H1
  - Basal: CON (90 nM) vs. MHD (45 nM)
  - 240 min: CON (45 nM) vs. MHD (90 nM)

- **G**: MG-H1
  - Basal: CON (3600 nM) vs. MHD (1800 nM)
  - 240 min: CON (1800 nM) vs. MHD (3600 nM)
Figure 2

Bar charts showing the levels of various cytokines and chemokines in the CON and MHD groups at baseline and 240 minutes. Cytokines include Fractalkine, IFNγ, IL10, IL17A, IL1β, IL6, IL8, MIP1α, and TNFα. The graphs indicate statistical significance with asterisks (*) and dagger symbols (†).
Figure 3

(A) RAGE
(B) OST48
(C) pIκBα
(D) TLR4
(E) TRIF

F: Western Blot Analysis

[Western Blot Images]
Figure 4

- **Fractalkine**
  - CON: ~0.02 pg/mg wet wt
  - MHD: ~0.04 pg/mg wet wt

- **IFNγ**
  - CON: ~0.01 pg/mg wet wt
  - MHD: ~0.03 pg/mg wet wt

- **IL10**
  - CON: ~0.2 pg/mg wet wt
  - MHD: ~0.22 pg/mg wet wt

- **IL17A**
  - CON: ~0.005 pg/mg wet wt
  - MHD: ~0.01 pg/mg wet wt

- **IL1β**
  - CON: ~0.05 pg/mg wet wt
  - MHD: ~0.1 pg/mg wet wt

- **IL6**
  - CON: ~0.0 pg/mg wet wt
  - MHD: ~0.4 pg/mg wet wt
  - *Note: MHD group has a significant increase compared to CON.*

- **IL8**
  - CON: ~0.23 pg/mg wet wt
  - MHD: ~0.46 pg/mg wet wt
  - †Note: MHD group has a significant increase compared to CON.*

- **MIP1α**
  - CON: ~0.03 pg/mg wet wt
  - MHD: ~0.06 pg/mg wet wt

- **TNFα**
  - CON: ~0.15 pg/mg wet wt
  - MHD: ~0.3 pg/mg wet wt
Figure Captions

**Figure 1** Plasma oxidation (panels A and B) and advanced glycation end products (panels C-G) in the basal state and post meal (+240 min) assessed via LC-MS/MS. See *Meal composition* in the Methods for more details regarding mixed meal nutrition information. CON, control subjects (n=8); MHD, maintenance hemodialysis patients (n=8); MetSO, methionine sulfoxide; AAA, amino adipic acid; CML, Nε-carboxymethyl lysine; CEL, Nε-carboxyethyl lysine; 3DG-H, 3-deoxyglucosone hydroimidazolone; G-H1, glyoxal hydroimidazolone-1; MG-H1, methylglyoxal hydroimidazolone-1; Data are mean ± SE; ^ Effect of time: \( P < 0.05 \); ^^ Effect of time: \( P < 0.001 \); * \( P < 0.001 \) vs CON; # \( P < 0.05 \) vs Basal.

**Figure 2** Plasma inflammatory biomarkers in the basal state and post meal (+240 min) assessed via multiplex. See *Meal composition* in the Methods for more details regarding mixed meal nutrition information. CON, control subjects (n=8); MHD, maintenance hemodialysis patients (n=8); IFNγ, interferon gamma; IL, interleukin; MIP1α, macrophage inflammatory protein 1 alpha; TNFα, tumor necrosis factor alpha; Data are mean ± SE; * Group effect: \( P < 0.05 \) vs CON; † Group effect: \( P < 0.10 \) vs CON.

**Figure 3** Biopsy-derived skeletal muscle (m. vastus lateralis) basal protein expression determined via Western blot of inflammatory receptors and downstream signaling components (A-E). Representative images of proteins assessed are depicted in panel F. CON, control subjects (n=8); MHD, maintenance hemodialysis patients (n=8); RAGE, receptor for advanced glycation end products; OST48, oligosaccharyltransferase
subunit 48; plkBα, phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; TLR4, Toll-like receptor 4; TRIF, TIR-domain-containing adapter-inducing interferon-β. RAGE bands (43 and 48 kDa) were quantified together. Protein expression were normalized to total protein (A, B, D, E) or total IκBα (C). Individual data points overlain on mean ± SE; * P < 0.05 vs CON.

**Figure 4** Biopsy-derived skeletal muscle (m. vastus lateralis) basal inflammatory cytokine protein expression determined via multiplex. Cytokine concentrations were normalized to mg muscle wet weight. CON, control subjects (n=8); MHD, maintenance hemodialysis patients (n=8); IFNγ, interferon gamma; IL, interleukin; MIP1α, macrophage inflammatory protein 1 alpha; TNFα, tumor necrosis factor alpha; Data are mean ± SE; * Group effect: P < 0.05 vs CON; † P < 0.10 vs CON.