

## **Supplementary Material**

Supplement to: Moradi H, Park C, Streja E, Argueta DA, DiPatrizio NV, You AS, Rhee CM, Vaziri ND, Kalantar-Zadeh K, Piomelli D. Association of Circulating Endocannabinoid Levels with Mortality in Patients on Maintenance Hemodialysis.

## Supplementary Methods

### Lipid Extraction and Analysis

Extraction and analysis of circulating ECBs were performed according to well-established and previously described methods.<sup>1-3</sup> Briefly, methanol (1 mL) containing internal standards [<sup>2</sup>H<sub>4</sub>]AEA (1 pmol) and [<sup>2</sup>H<sub>5</sub>]2AG (250 pmol) was added to patient serum (0.5 mL) and all lipids were extracted using chloroform (2 mL) and 0.1 M sodium chloride (0.5 mL). The organic phases were dried under N<sub>2</sub>, reconstituted in chloroform (2 mL) and underwent solid-phase extraction by application to open-bed silica gel columns to fractionate lipid groups based on polarity. Eluted fractions containing AEA and 2-AG (chloroform/methanol, 9:1, v/v) were again dried under N<sub>2</sub> and reconstituted in 120 µL of solvent mixture of chloroform and methanol (1:1, v/v) for UPLC/MS/MS analyses.<sup>4</sup>

### Analyses of 2-AG and AEA by UPLC/MS/MS

Lipids were analyzed using a Waters Acquity I-Class Ultra Performance Liquid Chromatography system coupled to a Waters TQS-micro Triple Quadrupole Mass Spectrometer. All samples were analyzed after one freeze-thaw cycle to avoid confounding by sample preparation. Lipids were separated using an Acquity UPLC BEH C18 column (50 x 2.1 mm; i.d. 1.7 µm), eluted by a gradient of methanol (0.25% acetic acid, 5mM ammonium acetate) in water (0.25% acetic acid, 5mM ammonium acetate) (from 80 to 100% methanol in 2.5 min, 100% 2.5-3.0 min, 100-80% 3.0-3.1 min) at a flow rate of 0.4 mL/min. Column temperature was kept at 40° C and samples were maintained in the sample manager at 10° C. Argon was used as collision gas (99.998% pure). 2-arachidonoyl-*sn*-glycerol (2-AG), anandamide (AEA), [<sup>2</sup>H<sub>5</sub>] 2-AG, and [<sup>2</sup>H<sub>4</sub>] AEA were identified in the positive ionization mode based on their retention times and

MS<sup>2</sup> properties and capillary voltage set at 0.1 kV. Cone voltage and collision energy as follows, respectively: 2-AG = 30v, 12v; [<sup>2</sup>H<sub>5</sub>] 2-AG = 25v, 44v; anandamide = 30v, 14v; [<sup>2</sup>H<sub>4</sub>] anandamide = 26v, 16v. ECBs were quantified using a stable isotope dilution method detecting protonated adducts of the molecular ions [M+H]<sup>+</sup> in the multiple reaction monitoring (MRM) mode. Acyl migration from sn-2 to sn-1 positions in monoacylglycerols is known to occur; thus, the sum of these isoforms (1-AG and 2-AG) was used as the concentration of serum 2-AG. Extracted ion chromatograms were used to quantify 2-AG ( $m/z = 379.2 > 287.26$ ) and AEA ( $m/z = 348.3 > 62.04$ ), and [<sup>2</sup>H<sub>5</sub>] 2-AG ( $m/z = 384.2 > 93.4$ ), and [<sup>2</sup>H<sub>4</sub>] AEA ( $m/z = 352.3 > 66.11$ ), which were used as internal standards (Cayman Chemical, Ann Arbor, MI, USA).<sup>5</sup>

## Supplementary Tables

**Table S1.** Unadjusted and adjusted Spearman's rank correlation coefficients (Rho) between serum 2-AG and data on laboratory, clinical, quality of life and depression tests in 400 maintenance hemodialysis patients.

| Variable  | Unadjusted |         | Adjusted* |         |
|---|------------|---------|-----------|---------|
|   | Rho        | P-value | Rho       | P-value |
| Laboratory tests                                |            |         |           |         |
| Hemoglobin (g/dL)                               | -0.10      | 0.06    | -0.07     | 0.20    |
| Iron saturation (%)                             | -0.07      | 0.23    | -0.03     | 0.61    |
| UIBC ( $\mu\text{g/dL}$ )                       | 0.15       | 0.005   | 0.16      | 0.005   |
| Lymphocytes (%)                                 | 0.03       | 0.59    | 0.03      | 0.64    |
| White blood cell ( $\times 1000 \text{ mm}^3$ ) | 0.08       | 0.14    | 0.08      | 0.16    |
| nPCR (g/kg/d)                                   | 0.02       | 0.75    | 0.08      | 0.15    |
| TBW (Watson) (l)                                | 0.10       | 0.07    | 0.18      | 0.001   |
| Weight (kg)                                     | 0.18       | 0.0007  | 0.19      | 0.0008  |
| Pre-dialysis weight (kg)                        | 0.19       | 0.0006  | 0.19      | 0.0006  |
| Post-dialysis weight (kg)                       | 0.19       | 0.0004  | 0.20      | 0.0004  |
| Quality of life measures                        |            |         |           |         |
| Physical functioning                            | -0.07      | 0.19    | -0.04     | 0.43    |
| Role limitations due to physical health         | 0.04       | 0.42    | 0.05      | 0.33    |
| Role limitations due to emotional problems      | -0.05      | 0.31    | -0.06     | 0.25    |
| Energy/fatigue                                  | -0.01      | 0.83    | -0.009    | 0.86    |
| Emotional well-being                            | 0.02       | 0.64    | 0.01      | 0.86    |
| Social functioning                              | 0.07       | 0.21    | 0.06      | 0.24    |
| Pain  | 0.01       | 0.78    | 0.04      | 0.50    |
| General health                                  | 0.004      | 0.94    | 0.009     | 0.86    |
| Physical health                                 | 0.00005    | 1.00    | 0.02      | 0.69    |
| Mental health                                   | -0.01      | 0.84    | -0.01     | 0.78    |
| Beck Depression Index score                     | -0.03      | 0.61    | -0.03     | 0.56    |

\*Results were adjusted for age, gender, race, ethnicity, diabetes and dialysis vintage.

Abbreviations: 2-AG, 2-arachidonoyl-*sn*-glycerol; nPCR, normalized protein catabolic rate; TBW, total body water (Watson formula); UIBC, unsaturated iron binding capacity.

**Table S2.** Unadjusted and adjusted Spearman's rank correlation coefficients (Rho) between serum AEA and data on laboratory, clinical, quality of life and depression tests in 436 maintenance hemodialysis patients.

| Variable  | Unadjusted |         | Adjusted* |         |
|---|------------|---------|-----------|---------|
|   | Rho        | P-value | Rho       | P-value |
| Laboratory tests                                |            |         |           |         |
| Hemoglobin (g/dL)                               | -0.02      | 0.68    | 0.03      | 0.60    |
| Iron saturation (%)                             | -0.11      | 0.04    | -0.05     | 0.36    |
| UIBC ( $\mu\text{g/dL}$ )                       | 0.02       | 0.65    | 0.01      | 0.82    |
| Lymphocytes (%)                                 | -0.11      | 0.04    | -0.12     | 0.03    |
| White blood cell ( $\times 1000 \text{ mm}^3$ ) | 0.06       | 0.26    | 0.06      | 0.29    |
| nPCR (g/kg/d)                                   | -0.17      | 0.0009  | -0.06     | 0.27    |
| TBW (Watson) (l)                                | 0.08       | 0.13    | 0.06      | 0.27    |
| Weight (kg)                                     | 0.09       | 0.10    | 0.02      | 0.76    |
| Pre-dialysis weight (kg)                        | 0.09       | 0.09    | 0.02      | 0.69    |
| Post-dialysis weight (kg)                       | 0.09       | 0.08    | 0.02      | 0.67    |
| Quality of life measures                        |            |         |           |         |
| Physical functioning                            | 0.03       | 0.55    | 0.11      | 0.03    |
| Role limitations due to physical health         | -0.004     | 0.94    | 0.04      | 0.43    |
| Role limitations due to emotional problems      | 0.006      | 0.91    | 0.03      | 0.57    |
| Energy/fatigue                                  | 0.02       | 0.73    | 0.03      | 0.51    |
| Emotional well-being                            | 0.10       | 0.05    | 0.10      | 0.05    |
| Social functioning                              | 0.05       | 0.36    | 0.07      | 0.19    |
| Pain  | -0.004     | 0.94    | 0.04      | 0.40    |
| General health                                  | 0.02       | 0.71    | 0.01      | 0.82    |
| Physical health                                 | 0.01       | 0.81    | 0.06      | 0.21    |
| Mental health                                   | 0.04       | 0.43    | 0.06      | 0.24    |
| Beck Depression Index score                     | -0.04      | 0.47    | -0.03     | 0.56    |

\*Results were adjusted for age, gender, race, ethnicity, diabetes and dialysis vintage.

Abbreviations: AEA, anandamide; nPCR, normalized protein catabolic rate; TBW, total body water (Watson formula); UIBC, unsaturated iron binding capacity.

**Table S3.** Incidence rates and hazard ratios (HR) with 95% confidence intervals (CI) for 12-month all-cause mortality according to serum 2-AG categories (reference, <32.5 pmol/mL) among 400 maintenance hemodialysis patients with 4 models of adjustment: (i) model 1 (unadjusted); (ii) model 2 (age, gender, race and ethnicity); (iii) model 3 (model 2 + diabetes and dialysis vintage); and (iv) model 4 (model 3 + IL-6).

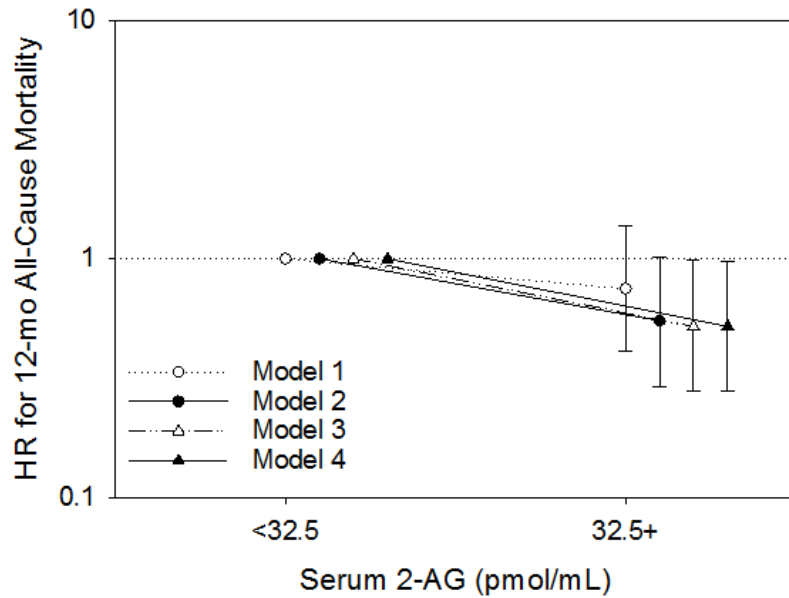
|                      |     |                    | 12-month All-Cause Mortality        |                   |                 |                   |                 |                   |                 |                   |         |
|----------------------|-----|--------------------|-------------------------------------|-------------------|-----------------|-------------------|-----------------|-------------------|-----------------|-------------------|---------|
|                      |     |                    | Model 1 (N=400)                     |                   | Model 2 (N=400) |                   | Model 3 (N=400) |                   | Model 4 (N=400) |                   |         |
| Serum 2-AG (pmol/mL) | N   | Mortality N (row%) | Mortality rate per 100 person-years | HR (95% CI)       | P-value         | HR (95% CI)       | P-value         | HR (95% CI)       | P-value         | HR (95% CI)       | P-value |
| <32.5                | 200 | 24 (12)            | 13.4 (8.0, 18.7)                    | Reference         |                 | Reference         |                 | Reference         |                 | Reference         |         |
| ≥32.5                | 200 | 19 (10)            | 10.1 (5.6, 14.7)                    | 0.75 (0.41, 1.37) | 0.35            | 0.55 (0.29, 1.02) | 0.06            | 0.52 (0.28, 0.99) | 0.04            | 0.52 (0.28, 0.98) | 0.04    |
| Total                | 400 | 43                 |                                     |                   |                 |                   |                 |                   |                 |                   |         |

**Table S4.** Incidence rates and hazard ratios (HR) with 95% confidence intervals (CI) for 12-month all-cause mortality according to serum AEA categories (reference, <1.06 pmol/mL) among 436 maintenance hemodialysis patients with 4 models of adjustment: (i) model 1 (unadjusted); (ii) model 2 (age, gender, race and ethnicity); (iii) model 3 (model 2 + diabetes and dialysis vintage); and (iv) model 4 (model 3 + IL-6).

|                     |     |                    |                                     | 12-month All-Cause Mortality |         |                   |         |                   |         |                   |         |
|---------------------|-----|--------------------|-------------------------------------|------------------------------|---------|-------------------|---------|-------------------|---------|-------------------|---------|
|                     |     |                    |                                     | Model 1 (N=436)              |         | Model 2 (N=436)   |         | Model 3 (N=436)   |         | Model 4 (N=436)   |         |
| Serum AEA (pmol/mL) | N   | Mortality N (row%) | Mortality rate per 100 person-years | HR (95% CI)                  | P-value | HR (95% CI)       | P-value | HR (95% CI)       | P-value | HR (95% CI)       | P-value |
| <1.06               | 217 | 19 (9)             | 9.5 (5.2, 13.8)                     | Reference                    |         | Reference         |         | Reference         |         | Reference         |         |
| ≥1.06               | 219 | 28 (13)            | 14.0 (8.8, 19.2)                    | 1.48 (0.83, 2.65)            | 0.19    | 1.07 (0.59, 1.95) | 0.83    | 1.07 (0.58, 1.97) | 0.84    | 0.91 (0.48, 1.72) | 0.77    |
| Total               | 436 | 47                 |                                     |                              |         |                   |         |                   |         |                   |         |

## Supplementary Figures

**Figure S1.** Association of serum 2-AG and 12-month all-cause mortality among 400 maintenance hemodialysis patients across 4 levels of adjustment: (i) model 1 (unadjusted); (ii) model 2 (age, gender, race and ethnicity); (iii) model 3 (model 2 + diabetes and dialysis vintage); and (iv) model 4 (model 3 + IL-6).

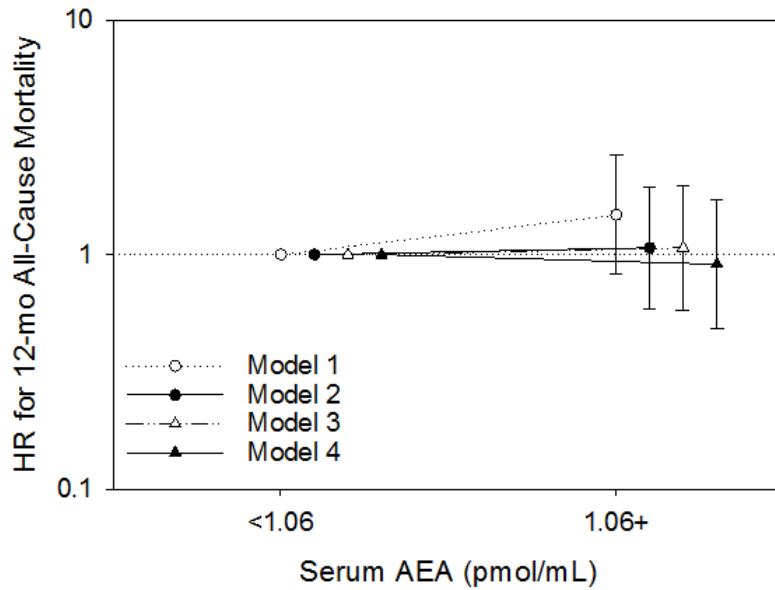


For visual purposes only, the y-axis was log-transformed.

Abbreviations: 2-AG, 2-arachidonoyl-*sn*-glycerol; HR, hazard ratio; mo, month.



**Figure S2.** Association of serum AEA and 12-month all-cause mortality among 436 maintenance hemodialysis patients across 4 levels of adjustment: (i) model 1 (unadjusted); (ii) model 2 (age, gender, race and ethnicity); (iii) model 3 (model 2 + diabetes and dialysis vintage); and (iv) model 4 (model 3 + IL-6).



For visual purposes only, the y-axis was log-transformed.  
Abbreviations: AEA, anandamide; HR, hazard ratio; mo, month.

## Supplementary Material References

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