**Urine APOL1 isoforms reflect plasma derived liver-synthesized proteins**

**Supplementary Materials**

**Detailed Methods**

***ApoL1 specific magnetic bead preparation***

APOL1-specific monoclonal antibodies (cat no 66124-1-Ig, 1.573 mg/mL, purchased from Proteintech Manchester, United Kingdom) were coated to magnetic beads (Dynabeads™ M-280 Sheep Anti-Mouse IgG, cat no 11201D, ThermoFischer). The protocol included Dynabeads™ resuspended in vials by vortex for 1 minute with 1 mL transferred to a 1.5 mL Eppendorf tube placed in a DynaMag™-2 Magnet (ThermoFisher) for 2 minutes to remove the supernatant. Beads were washed by resuspension in 1 mL dPBS (Gibco dPBS wo Ca/Mg, Cat no 14190-144, ThermoFischer) containing 0.1 % BSA (cat no A1595, Sigma Life Sciences) and the supernatant was discarded using the magnet. Beads were resuspended in 1 ml dDPBS containing 0.1% BSA and 50µL of the APOL1-specific monoclonal antibody was added to the bead suspension. After 24 hours of incubation at +4°C with tilt rotation using a HulamixerTM-Sample mixer, the Eppendorf tube was placed on the magnet and the supernatant discarded. Beads were washed twice using 1 mL dPBS containing 0.1% BSA and once using 4 mL PBS (BupH™ Phosphate Buffered Saline Packs, cat no 28372, ThermoFischer, dissolved in MQ water according to the instruction). The tube was placed on the magnet for 2 minutes after each wash to discard the supernatant. The APOL1-specific antibodies were subsequently cross-linked to the beads by resuspension in 5 mL of freshly made 5mM BS3 (bis(sulfosuccinimidyl)suberate) No-Weigh™ Format, cat no A39266, ThermoFischer) in PBS, followed by incubation at room temperature with tilt rotation. After 30 minutes, the crosslinking was quenched by addition of 250µL 1M Tris-HCl (1M Tris-Hydrochloride buffer solution pH 7.5, cat no 10123722, ThermoFischer) and subsequent incubation with tilt rotation for 15 minutes. Beads were washed three times using 4 mL dPBS containing 0.05% Tween-20 (cat no P2287, Sigma Life Sciences) and the DynaMag™-2 Magnet to discard the supernatant after each wash. Beads were resuspended in 1 ml dPBS containing 0.1% BSA and 3mM sodium azide (cat no 08591, Sigma LifeSciences) for long term storage at +4°C.

***Ultrafiltration***

Prior to the immunoprecipitation, an ultrafiltration procedure for concentration and buffer exchange of the urine was performed using a protein purification unit from Amicon with a 10 kD cut-off ultrafilter (Amicon Ultra-2 Centrifugal Filter unit UFC201024 EMD Millipore). The Amicon unit was prewashed to minimize nonspecific binding, by 0.5 mL TBS containing 0.1% Tween 20 followed by centrifugation at 4,000 × g for 5 minutes at +20°C using a swinging-bucket rotor. Thawed urine in 1 mL aliquots (5 –12 ml per individual) was cleared from solids by centrifugation at 10,000 x g for 20 minutes at +6°C in a microcentrifuge and 950 µL of the supernatant was carefully transferred to the Amicon unit. After centrifugation at 4,000 × g for 15 minutes at +20°C, the concentrated urine was buffer-exchanged by addition of 1.5 mL dPBS and centrifugation for additional 30 minutes. The concentrated and buffer-exchanged urine was recovered by reversed spin into the collection tube by centrifugation at 4,000 x g for 5 minutes at +6°C. The volume of the recovered ultrafiltrate from each individual was measured and stored at -80°C until immunoprecipitation. 30 µL of the pre-cleared and cleared urine was stored at -80°C for APOL1 quantification using the ELISA described below.

***Immunoprecipitation***

The APOL1-specific cross-linked beads prepared above were re-suspended in the Eppendorf tube by vortex for 30 seconds and 10 µL transferred to a 1.5 mL Eppendorf tube placed in the [DynaMag™-2 Magne](https://www.thermofisher.com/order/catalog/product/12321D)t to remove the supernatant. The beads were washed using 100 µL dPBS containing 0.1% BSA. The supernatant was discarded using the magnet and the beads were resuspended in 10 µL dPBS containing 0.1% BSA. The ultrafiltrated urine sample (pooled for each individual) was thawed for 5 minutes in a tap water bath and 11 µL was saved for subsequent ELISA quantification. The washed beads were placed in the magnet to remove the supernatant and the remainder of the thawed ultrafiltrate was mixed with the beads using a vortex for 5 seconds and subsequent incubation for 60 minutes at room temperature by tilt rotation using the HulamixerTM. The tube was placed in the magnet for 2 minutes and the supernatant removed and stored for subsequent ELISA quantification to calculate the amount of APOL1 captured by the breads. The beads were washed tree times using 150 µL ammonium bicarbonate and the tube placed in the magnet after each wash to remove the supernatant. The supernatant from the three wash cycles (total 450 µL) was saved for subsequent ELISA quantification to calculate the APOL1 loss from the beads during the wash procedure. The binding efficiency of urine APOL1 on beads was determined as: IP efficacy = ((APOL1 prior to IP)-(not bound to beads)-(lost during wash)) / (APOL1 prior to IP)\*100.

 The APOL1 captured on the beads was eluted using 0.1% RapiGest™ surfactant in ammonium bicarbonate as follows; 25 µL 0.1% RapiGest was added to the Eppendorf tube containing the APOL1 captured beads and incubated for 2 minutes while mixing for 5 seconds every 30 seconds during the incubation. The Eppendorf tubes was placed in the magnet and the supernatant with the eluted APOL1 was collected. 25 µL 0.1% RapiGest was again added to the beads and the procedure was repeated. The eluate was pooled (50 µL) and stored in Non-stick Microfuge tubes (AM12350, ThermoFisher) at - 80°C awaiting LC-MS/MS analysis.

**APOL1 *quantification by ELISA***

APOL1 from the pre-cleared urine, cleared urine, ultrafiltrate, immunoprecipitation supernatant and immunoprecipitation wash was quantified using a commercial sandwich ELISA kit from Proteintech (Catalogue #KE00047) with an extended standard calibration curve to cover a range of 41-10,000 ng/mL. All samples, including calibration samples, were analyzed in duplicate. The response of the standard curve was plotted as absorbance units on the linear scale versus concentration on the log scale and the 4-parameter function was used for curve fitting. The acceptance criteria for each analysis were based on the performance of the calibration samples; five of the back-calculated results for the calibration standards should be within 20% of their target concentration (25% at limit of quantification) with an imprecision of <25% of the duplicate values.

The pre-cleared and cleared urine samples were diluted 8-fold and the ultrafiltrate and immunoprecipitation supernatant were diluted 20-fold prior to analysis. The immunoprecipitation wash was analyzed undiluted.

***Chemicals and reagents used for LC-MS/MS assay***

Stable isotope-labeled internal standard protein epitope signature tags (SIS PrESTs) were purchased from Atlas Antibodies AB (Stockholm, Sweden). Stable isotope 13C and 15N were incorporated into the carboxyl terminus of lysine and arginine, giving mass shifts of 8 Da with lysine and 10 Da with arginine. The isotopic incorporation was >99.4%. The amino acid sequences of the SIS PrESTs are depicted in **Supplementary Table S1**. RapiGest™ surfactant used for elution of immuno-precipitated APOL1 was purchased from Waters Corporation (USA). Ammonium bicarbonate (AMBIC), 2-chloroacetamide (CAA), trifluoroacetic acid (TFA), phosphate-buffered saline (PBS) and SOLu-Trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (FA) and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Thermo Scientific. The 96-well low-binding PCR microplates were purchased from Eppendorf (Germany), the associated sealing mat (Axygen™ AxyMats™ Sealing Mat for 96 Well PCR Microplates) and acetonitrile (AcN) was purchased from Fisher Scientific.

***LC-MS/MS Assay Development and Optimization using the SIS PrESTs***

The digestion procedure was adapted from a previous publication [1]. Briefly, the three SIS PrESTs were digested separately to create a peptide mixture used to identify proteotypic peptides for each APOL1 genotype and to optimize their corresponding SRM transitions. 50 pmol of each SIS PrEST was spiked in to 50 mm AMBIC buffer. TCEP was added to a final concentration of 5 mm and samples were incubated for 60 min at 60°C. CAA was added to a final concentration of 15 mm and samples were incubated in darkness for 30 min. Samples were digested overnight at 37°C by addition of 1 μg trypsin. The enzymatic reaction was stopped by addition of formic acid to a final concentration of 1%. The tryptic peptide solution was then dried down using a sample concentrator (miVac, Genevac Ltd, Ipswich, UK). Samples were resuspended in 100 μL of 0.1% formic acid and subsequently transferred to 300 μL conical polypropylene autosampler vials (Agilent Technologies; Santa Clara, CA) for LC-MS/MS analysis. Approximately 1 pmol of each SIS PrEST was injected onto the LC-MS system. Three MRM transitions for each proteotypic peptide corresponding to the different APOL1 genotypes were evaluated and the collision energies were optimized. The transitions that gave the strongest signal-to-noise ratios (S/N) for the genotype specific peptide sequences and their corresponding optimized collision energies as well as their chromatographic retention times are listed in Supplementary Table S2. Since the peptide used to detect the G1 variant contains methionine, which is an amino acid prone to oxidation, transitions that monitor oxidation of methionine were included in the MS/MS method.

***Tryptic digestion and desalting of ultra filtrated and immunoprecipitated urine samples***

50 μL of immuno-precipitated eluate (0.1% RapiGest) was spiked with the equimolar mixture of the SIS PrESTs corresponding to 100 fmol. Then, 100 mM TCEP was added to a final concentration of 8.3mM and the sample was reduced by incubation for 60 min at 60°C in a Thermo mixer (Eppendorf) at 700 rpm. Alkylation was performed by addition of 200mM CAA to a final concentration of 28.6 mM and incubation in darkness for 30 minutes. The sample was adjusted to pH 8 by addition of 50 mM Tris buffer (pH 8) and subjected to tryptic digestion for 16 h by addition of 4 g of trypsin performed at 37°C in a Thermo mixer at 700 rpm. The digestion was stopped by quenching the samples with FA (final concentration 1%). The digested samples were subjected to solid phase extraction (SPE) that was performed using the Bravo AssayMAP liquid handler robot (Agilent Technologies) using reversed-phase cartridges (RPS, #65496–60033, Agilent Technologies). The cartridges were activated with 100 μL of 0.1% TFA in 50% AcN using a flow rate of 300 μL/min. The cartridges were equilibrated with 50 μL 0.1% TFA in water using a flow rate of 100 μL/min. The trypsinated samples were loaded onto the cartridge using a flow rate of 10 μL/min. The cartridge was then washed with 50 μl of 0.1% TFA in water using a flow rate of 100 μL/min. The syringe was washed with 50 μL 0.1% TFA in 50% AcN using a flow rate of 300 μL/min and subsequently the samples were eluted in 20 μL of 0.1% TFA in 70% ACN using a flow rate of 5 μL/min. The peptides were eluted into a lo-bind PCR plate (Eppendorf). Finally, the SPE eluates were dried down in a concentrator (miVac, Genevac Ltd, Ipswich, UK) and re-constituted in 20 μL of 2% AcN, 0.1% FA. The entire volume of these samples was injected onto the LC-MS system.

The LC-SRM/MS assay detected one specific tryptic fragment per isoform. The following isoform specific peptides were monitored: LNILNNNYK (wild type), LNMLNNNYK (G1), and LNILNNK (G2). These proteotypic peptides and their corresponding SRM transitions are included in **Supplementary Tables S1** and **S2**, below.

**Supplementary Table S1. Amino acid sequences of the stable isotope labelled recombinant protein fragments (SIS PrEST standards) where the bold sequences represent proteotypic peptides for the APOL1 isoform variants.**

|  |  |  |
| --- | --- | --- |
| SIS PrEST  | Mw (Da) | Sequence |
| 40034 (G0) | 30063.6 | RANLQSVPHASASRPRVTEPISAESGEQVERVNEPSILEM SRGVKLTDV APVSFFLVLDVVYLVYESKHLHEGAKSETAEELKKVAQELEEK**LNILNNNYK**ILQADQEL |
| 40035 (G1) | 30081.6 | RANLQSVPHASASRPRVTEPISAESGEQVERVNEPSILEMSRGVKLTDVAPVSFFLVLDVVYLVYESKHLHEGAKSETAEELKKVAQELEEK**LNMLNNNYK**ILQADQEL |
| 40036 (G2) | 29786.3 | RANLQSVPHASASRPRVTEPISAESGEQVERVNEPSILEMSRGVKLTDVAPVSFFLVLDVVYLVYESKHLHEGAKSETAEELKKVAQELEEK**LNILNNK**ILQADQEL |

**Supplementary Table S2. SRM transitions monitored for determination of APOL1 isoform variants by mass spectrometry. aCollision energy (arbitrary units); bRetention time (min).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ApoL1 variants | Precursor m/z | Product m/z | CEa | RTb |
| LNILNNNYK (G0) | 553.3 | 765.4652.3992.5 | 16 | 9.8 |
| LNILNNNY[K-13C615N2] | 557.3 | 773.4660.31000.5 | 16 | 9.8 |
| LNMLNNNYK (G1) | 562.3 | 896.4765.4652.3 | 16 | 8.5 |
| LNMLNNNY[K-13C615N2] | 566.3 | 904.4773.4660.3 | 16 | 8.5 |
| LNILNNK (G2) | 414.8 | 601.4488.3601.4 | 10 | 8.3 |
| LNILNN[K-13C615N2] | 418.8 | 609.4496.3609.4 | 10 | 8.3 |

***LC-MS/MS Analysis***

The samples were injected onto the LC system (Agilent 1290, Waldbronn, Germany) and separated using a Zorbax Eclipse XDB-C18 column (2.1 × 150 mm) packed with 1.8 μm particles with a pore size of 80 Å (Agilent Technologies), which was interfaced to a Triple Quadrupole mass spectrometer (Agilent 6490, Agilent Technologies) using the Agilent Jet Stream flow ESI source. For the initial screening, an unscheduled method with a dwell time of 30 ms for all target specific transitions was used. The gradient started at 97% solvent A (0.1% FA, H2O) and 3% solvent B (0.1% FA, AcN) with a constant flow rate of 0.400 mL/min. Peptides were eluted with a linear increase of solvent B from 3–38% over 15 min, followed by a 1 min washout as the percentage of solvent B was increased to 80%. The column was then re-equilibrated for 3 min with 3% of solvent B. The temperature of the column compartment was set to 50 °C and the autosampler temperature was maintained at 6 °C.

The immuno-precipitated samples were analyzed using a Dynamic MRM method where the dwell time is set automatically using a cycle time of 0.5 seconds. The MS parameters of the Agilent 6490 triple quadrupole mass spectrometer were: positive ion mode, 3.5 kV capillary voltage, nozzle voltage 300 V, drying gas flow rate 15 L/min at 150 °C, nebulizer gas pressure 30 PSI at 250 °C. Q1 and Q3 were set to unit resolution. **Supplementary Figure S1** displays urine APOL1 concentration, by recipient *APOL1* genotype. **Supplementary Table S3** displays APOL1 isoforms detected in the urine of transplant recipients.

**Supplementary Table S3. APOL1 isoform detection in kidney transplant recipient urine samples**

|  |  |
| --- | --- |
|  | **Urine APOL1 Isoform Detected** |
| **Transplant Recipient** | **Recipient *APOL1*** **Genotype** | **Donor *APOL1* Genotype** | **Donor** | **Recipient** |
| 1 | G1/G2 | G0/G0 | ND | Yes |
| 2 | G1/G2 | G0/G0 | ND | Yes |
| 3 | G1/G2 | G0/G0 | ND | ND  |
| 4 | G1/G2 | G0/G0 | ND | Yes |
| 5 | G1/G2 | G0/G0 | ND | Yes |
| 6 | G1/G2 | G0/G0 | ND | Yes |
| 7 | G1/G2 | G0/G0 | ND | Yes |
| 8 | G1/G1 | G0/G0 | ND | Yes |
| 9 | G1/G1 | G0/G0 | ND | ND |
| 10 | G1/G1 | G0/G0 | ND | ND |
| 11 | G1/G1 | G0/G0 | ND | Yes |
| 12 | G1/G1 | G0/G0 | ND | Yes |
| 13 | G1/G1 | G0/G0 | ND | ND |
| 14 | G1/G1 | G0/G0 | ND | ND |
| 15 | G1/G1 | G0/G0 | ND | Yes |
| 16 | G1/G1 | G0/G0 | ND | ND |
| 17 | G1/G1 | G0/G0 | ND | ND |
| 18 | G1/G1 | G0/G0 | ND | Yes |
| 19 | G2/G2 | G0/G0 | ND | ND |
| 20 | G2/G2 | G0/G0 | ND | Yes |
| 21 | G2/G2 | G0/G0 | ND | Yes |
| 22 | G2/G2 | G0/G0 | ND | Yes |
| 23 | G2/G2 | G0/G0 | ND | Yes |
| 24 | G2/G2 | G0/G0 | ND | Yes |
| 25 | G0/G0 | G1/G1 | Yes\* | Yes |
| 26 | G0/G0 | G2/G2 | ND | Yes |

**ND: not detected; \*Subject with very high urine APOL1 concentration**

**Supplementary Figure S1. Urine APOL1 concentration, by recipient *APOL1* genotype**

Urine APOL1 protein concentrations were determined in recipients of high and low risk APOL1 transplant kidneys using an ELISA quantification assay. The assay LOQ was 0.8 ng/mL.



**Reference:**

1. Hober A, Edfors F, Ryaboshapkina M, Malmqvist J, Rosengren L, Percy AJ, Lind L, Forsström B, Uhlen M, Oscarsson J, and Miliotis T. (2019) Absolute Quantification of Apolipoproteins Following Treatment with Omega-3 Carboxylic Acids and Fenofibrate Using a High Precision Stable Isotope-labeled Recombinant Protein Fragments Based SRM Assay. MCP 18(12), 2433-2446.