**APOL1 Risk Variants Impair Multiple Mitochondrial Pathways in a Metabolomics Analysis**

Lijun Ma, Nicholette D. Palmer, Young A Choi, Mariana Murea, James A. Snipes, John S. Parks, Carl D. Langefield, and Barry I. Freedman

Abstract

**Background** Kidney risk variants (KRVs) in the APOL1 gene are associated with mitochondrial dysfunction. However, the molecular spectrum of metabolites affected by the G1 and G2 KRVs, and the downstream mitochondrial pathways they affect, remain unknown.

**Methods** We performed a metabolomics analysis using HEK293 Tet-on cells conditionally expressing APOL1 G0, G1, and G2 KRVs to determine the patterns of metabolites and pathways potentially involved in nephropathy. The Welch two-sample t test, matched-pairs t test, and two-way repeated measures ANOVA were used to identify differential metabolites. Random forest, a supervised classification algorithm that uses an ensemble of decision trees, and the mean-decrease-accuracy metric were applied to prioritize top metabolites.

**Results** Alterations in the tricarboxylic acid cycle, increased fatty acid oxidation, and compromised redox homeostasis were the major pathways affected by overexpression of APOL1 KRVs.

**Conclusions** Impairment of mitochondrial membrane respiratory chain complex I appeared to account for critical metabolic consequences of APOL1 KRVs. This finding supports depletion of the mitochondrial membrane potential, as has been reported.


Introduction

Two common coding variants in the APOL1 gene are associated with development of CKD in populations with recent African ancestry: G1 (two variants, S342G and I384M, in nearly perfect linkage disequilibrium) and G2 (deletion of amino acids N388 and Y389) (1). Endogenous and locally acting, but not circulating, APOL1 protein is believed to cause nephropathy, on the basis of data from kidney transplantation, cell biology, and animal models (2–5).

Multiple pathways, including enhanced autophagic cell death, pyroptosis, and altered endo-lysosomal trafficking, have been implicated in the cellular injury induced by APOL1 kidney risk variants (KRVs) (5,6). Recently, Ma et al. (7) and Granado et al. (8) independently reported that the APOL1 G1 and G2 KRVs reduced mitochondrial function. Shah et al. (9) subsequently reported that APOL1 KRVs induced cell death via opening of the inner mitochondrial membrane permeability transition pore (mPTP). APOL1 KRVs were also found to induce mitochondrial fission, resulting in depletion of the mitochondrial membrane potential (10). APOL1 C-terminal variants caused extensive organelle fission, including mitochondrial fission, in podocytes (11). Hence, mounting evidence supports a key role for mitochondrial dysfunction in development of APOL1 nephropathy.

Mitochondria are a rich source of signature metabolites. As such, this manuscript reports a systematic, metabolomics approach to detecting pathways whereby APOL1 KRVs may be injurious to cells. We used doxycycline (Dox)-inducible human embryonic kidney (HEK293) Tet-on cells that conditionally express APOL1 G0 (reference), and the G1 and G2 KRVs (10). The goal was to understand metabolic changes elicited by overexpression of APOL1 G1 and G2 in HEK293 Tet-on cells and elucidate potential underlying mechanisms.

Materials and Methods

**Culture of HEK293 Tet-On Cell Lines and Collection of Cells for Metabolomics Analysis**

HEK293 Tet-on APOL1 reference G0, G1, G2, and empty vector cells were established, as reported (12). Identical study designs and conditions were used as in prior experiments, with cells of each genotype/treatment undergoing experiments performed in triplicate (10). In brief, 5–10 ng/ml of Dox induction (final concentration) was applied for no more than 16 hours to ensure comparable APOL1 expression levels, without
changes in cell viability, on the basis of the lactate dehydrogenase release assay (10). The cell culture medium was removed and cells gently washed with PBS. After removal of the PBS wash buffer, cells were resuspended in PBS and counted to determine total numbers using a hemocytometer. Cell pellets were formed with centrifugation at 500 × g for 5 minutes. Supernatant was removed and cell pellets were stored in 1.5 ml centrifuge tubes at −80°C for metabolomics analyses. Supplemental Figure 1 displays the study design, including a summary of the sample layout.

Technical Platforms for the Metabolomics Study

Samples for the metabolomics analysis were prepared using the automated MicroLab STAR system (Hamilton Company, Franklin, MA) in the Metabolon, Inc. (Morrisville, NC) research laboratory. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and recover chemically diverse metabolites, proteins were precipitated with methanol while undergoing vigorous shaking for 2 minutes (GenoGrinder 2000; Glen Mills, Clifton, NJ). This was followed by centrifugation at 12,000 × g for 2 minutes. The resulting extract was divided into five fractions; two were analyzed by different reverse-phase, ultra-high performance liquid chromatography–tandem mass spectroscopy (reverse-phase UPLC-MS) methods with positive-ion-mode electrospray ionization (ESI), one was analyzed by reverse-phase UPLC-MS/MS with negative-ion-mode ESI, and one was analyzed by hydrophilic-interaction liquid chromatography/UPLC-MS with negative-ion-mode ESI. The fifth fraction was kept as a backup. Samples were briefly placed on a TurboVap (Zymark, Hopkinton, MA) to remove the organic solvent. Sample extracts were stored overnight in liquid nitrogen before being prepared for analysis. The informatics system comprised four components: the Laboratory Information Management System, data extraction and peak-identification software, data processing tools for quality control (QC) and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

Metabolite Quantification and Data Normalization

Raw data were extracted, peaks identified, and QC processing was performed based on a website-service platform using Microsoft’s .NET technologies. Peaks were quantified on the basis of the area under the curve. Biochemical data for metabolites were normalized to total protein, determined by Bradford assay, and cell counts to account for differences in metabolite levels due to variation in the amount of material in each sample. Missing metabolite data were imputed to the lowest observed value for that compound in the experiment.

Total RNA Isolation, QC, and Real-Time PCR for Key Subunits of Complex I

Total RNA was isolated from HEK293 Tet-on cells using RNAeasy Mini Kit (Qiagen, Hilden, Germany). The quantity and quality of isolated RNA were determined by ultraviolet spectrophotometry and electrophoresis, respectively, on the Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

To determine transcript levels of complex I key subunits in cells, 200 ng of RNA was reverse transcribed with random hexamer primers using the TaqMan RT Kit (Applied Biosystems, Foster City, CA). Primers were designed to capture known splice variants of N module core subunits NDUFV2 (V2_forward, 5'-CTCCATTGTATTTCCACCA-3'; V2_reverse, 5'-TTCAAATCGGCCTTGCATTAT-3') and NDUFSL (S1_forward, 5'-TTTGAAGCCACACTTTATATG-3'; S1_reverse, 5'-ATTGCTGTCCATCATTCTTT-3') of complex I. Real-time PCR in the presence of SYBR Green was performed with a Roche 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany) using 18S ribosomal RNA for normalization; primer sequences were described previously (13). The ΔΔCt method (14) was used to quantify the relative levels of mRNA. Fold changes were normalized to mRNA levels on HEK293 Tet-on G0 cells without Dox induction. All experiments were performed in triplicate.

Statistics and Bioinformatics Analyses

Two types of statistical analyses were performed: hypothesis testing and classification analysis. Analyses compared the metabolite profiles in G0 cells (reference) with those in G1 and G2 cells. Standard statistical analyses were performed in ArrayStudio on log-transformed data. For analyses not typically run in ArrayStudio, the programs R (http://cran.r-project.org/) or JMP (http://www.jmp.com) were used.

The Welch two-sample t test, matched-pairs t test, and two-way repeated measures ANOVA were used to identify differential metabolites. P values <0.05 were used to identify metabolites for implicated pathways. An estimate of the false discovery rate (q value) was calculated to consider the multiple comparisons that occur in metabolomics-based studies.

Random forest, a supervised classification algorithm that uses an ensemble of decision trees, and the mean-decrease-accuracy metric was applied to prioritize top metabolites using R (https://www.r-project.org/) (15). The top 30 metabolites in the list were the output and used for further investigation of potential pathways.

Results

Metabolite Summary and Basic Comparison Analysis

After initial normalization, metabolite profiles were determined from the experimental groups as raw data (Supplemental Table 1). The dataset comprised 692 total metabolites, 645 of known identity (named biochemicals) and 47 of unknown structural identity (unnamed biochemicals). After additional normalization for cell number, log transformation, and imputation of missing values (if any), the minimum observed value for each metabolite was reported (Supplemental Table 2). ANOVA was used to identify metabolites that differed significantly among cells expressing APOL1 G0 (control) versus G1 and G2 KRVs. Metabolites that achieved nominal statistical significance (P <0.05), and those trending toward significance (0.05 <P <0.10) are
shown in Supplemental Table 3. Two-way ANOVA identified metabolites exhibiting significant interaction and main effects for treatment (±Dox) and genotype (G0, G1, G2).

Pathways Implicated by Classification Analyses of Metabolites

When the random-forest analysis was performed comparing the four Dox-induced HEK293 Tet-on cell lines (including empty vector), results provided a predictive accuracy of 83%, well above random chance (25% given four groups; Figure 1). This suggested the top 30 metabolites identified among the genotypic groups could underlie phenotypic differences. Top-ranked metabolites were primarily those involved in carbohydrate and lipid metabolism.

As shown in Figure 2, G1- and G2-expressing cell lines (versus G0) showed significant increases in tricarboxylic acid (TCA) cycle intermediates, including acetyl-CoA, citrate, and aconitate; accompanied by significant decreases in downstream TCA intermediates, such as succinate, succinylcarnitine (correlated with succinyl-CoA), and fumarate in G1 cells, and α-ketoglutarate, succinylcarnitine, and fumarate in G2 cells. These data could reflect impairment in the TCA cycle, particularly the step(s) between citrate and α-ketoglutarate. This may lead to accumulation of the upstream molecules (acetyl-CoA, citrate, aconitate) and depletion of downstream molecules.

Increased fatty-acid oxidation was also evident in metabolites from APOL1 G1 and G2 cell lines (versus G0). Fatty acids are activated for degradation by conjugation with CoA in the cytosol. Figure 3A shows long-chain fatty acids destined for oxidation are conjugated to carnitine by the outer mitochondrial membrane carnitine palmitoyltransferase 1 (CPT1); this results in release of CoA. The acylcarnitines are transported across the intermembrane space by carnitine/acylcarnitine translocase, and are acted upon by the inner membrane CPT2 to release carnitine and reconjugate long-chain fatty acids with CoA for mitochondrial fatty acid β-oxidation. Figure 3B shows multiple long-chain acylcarnitines were significantly reduced in G1 and G2 cells—including palmitoyl carnitine (C16:1), eicosanoylcarnitine (C20:1), linoleoylcarnitine (C18:2), and lignoceryl carnitine (C24)—whereas acetyl-CoA was increased; this suggests an increase in β-oxidation (16).

Changes in redox homeostasis were also observed. Figure 4A shows that γ-glutamyl (GG) amino acids are formed by the activity of the cell-surface enzyme family GG transpeptidase. These amino acid–gluthathione conjugates traverse the cell membrane, release the amino acid intracellularly, and regenerate glutathione through the 5-oxopropionate intermediate. Multiple GG amino acids were significantly decreased in G1- and G2-expressing cells (Figure 4B), which may reflect increased demand for glutathione regeneration and detoxification of reactive oxygen species. Consistent with this observation, markers of oxidative stress (methionine sulfone and methionine sulfoxide) (17) were significantly elevated in G1 and G2 cells, respectively (Figure 4C).

Altered one-carbon metabolism and transsulfuration were observed. Figure 5 shows that methionine is critical to many key methylation reactions in cellular metabolism. Under some circumstances, homocysteine can be diverted...
along the transsulfuration pathway to produce cysteine, a precursor to the antioxidant glutathione and taurine (18). Several intermediates in this pathway were elevated in G1 cells, including methionine, S-adenosyl methionine, S-adenosylhomocysteine, cystathionine, and taurine, whereas cysteine was reduced in G1 and G2 cells.

Downregulation of Complex I Core Subunits NDUFV2 and NDUFS1

As shown in Figure 6, NDUFV2 and NDUFS1 transcript levels were downregulated in G1 and G2 HEK293 Tet-on cells after Dox-induced mild APOL1 expression, compared with those without Dox (all \( P < 0.05 \)).

Discussion

This metabolomics analysis focused on the identification of metabolic pathways in HEK293 cells expressing APOL1 G1 and G2 KRVs, relative to G0. The top 30 metabolites were prioritized to narrow down pathways most likely contributing to KRV effects. The data for each relevant metabolite was displayed by genotype and cell treatment in jittered dot plots to simplify presentation. Metabolomics revealed alterations in the TCA cycle, increased fatty-acid oxidation, and compromised redox homeostasis as major pathways affected by overexpression of APOL1 KRVs.

Metabolomics revealed that an altered TCA cycle was the most prominent change observed with expression of APOL1 KRVs. Succinate was the top known metabolite in the analysis. Interestingly, several metabolites in the TCA cycle were significantly decreased in G1- or G2-expressing cells, and others were significantly increased. When intermediate metabolites in the TCA cycle are visualized as substrates and products of enzymatic catalysis (Supplemental Figure 2), the results were compatible with a reduction in the conversion of citrate to \( \alpha \)-ketoglutarate (downstream TCA metabolites) and accumulation of upstream metabolites, citrate and acetyl-CoA. This most likely resulted from
A  Indicators of increased fatty acid oxidation

![Diagram of fatty acid oxidation]

B

<table>
<thead>
<tr>
<th>Sub Pathway</th>
<th>Biochemical Name</th>
<th>Fold of Change (with DOX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acid Metabolism</td>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>CPT1</td>
<td>acetyl-CoA</td>
<td>6.41</td>
</tr>
<tr>
<td>Fatty Acid Metabolism (Acyl Carnitine, Monounsaturated)</td>
<td>myristoylcarnitine (C14)</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>palmitoylcarnitine (C16)</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>palmitoleoylcarnitine (C16:1)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>stearoylcarnitine (C18:0)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>arachidoylcarnitine (C20:0)</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>lignoceroylcarnitine (C24:0)</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>cerotolylcarnitine (C26:0)</td>
<td>0.99</td>
</tr>
<tr>
<td>Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)</td>
<td>myristoylcarnitine (C14:1)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>palmitoylcarnitine (C16:1)</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>oleoylcarnitine (C18:1)</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>eicosanoylcarnitine (C20:1)</td>
<td>0.58</td>
</tr>
<tr>
<td>Fatty Acid Metabolism (Acyl Carnitine, Polyunsaturated)</td>
<td>linoleoylcarnitine (C18:2)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>arachidonoylcarnitine (C20:4)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Figure 3. | APOL1 kidney risk variants increase fatty acid oxidation. Mild overexpression of APOL1 G1 and G2 variants: (A) reduced acyl carnitine and increased acetyl-CoA, suggesting activated fatty-acid β-oxidation; (B) reduced levels of long-chain saturated, monounsaturated, and polyunsaturated acyl-carnitines, whereas acetyl-CoA was significantly increased. Table cells in green indicate significant differences (P<0.05) between groups, metabolite ratio of <1.00; light green trended toward statistical significance (0.05<P<0.10), metabolite ratio <1.00; red indicates significant between-group differences (P<0.05), metabolite ratio ≥1.00. CPT1, carnitine-palmitoyltransferase 1; CPT2, carnitine-palmitoyltransferase 2.

Reduced nicotinamide adenine dinucleotide hydrate (NADH): ubiquinone oxidoreductase (type-I NADH dehydrogenase or mitochondrial complex I) activity, because it negatively affects isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Supplemental Figure 2), and expression levels of complex I N module core subunits were significantly reduced (Figure 6).

Mitochondrial complex I is the first large protein complex in the respiratory chain that catalyzes electron transfer from NADH to coenzyme Q10 and translocates protons across the inner mitochondrial membrane (19). Human mitochondrial complex I contains 45 subunits in mammals (20). As we reported (7), APOL1 KRV-induced mitochondrial dysfunction was characterized by decreased expression of transcripts encoding key enzyme subunits of complexes I, II, III, IV, and V in the mitochondrial respiratory chain, especially complex I subunits (7). It is striking that APOL1 G1 and G2 KRVs broadly affect numerous proteins that form a single complex by regulation of gene expression at the level of transcription. Regulation of enzymes and their subunit proteins is highly specific (21). Therefore, we feel it is less likely that APOL1 KRVs regulate structurally divergent enzymes and their subunits; they are more likely to affect a key biologic event or process that triggers downstream effects. APOL1 KRVs reportedly deplete mitochondrial membrane potential via fission (10) and/or opening of the inner mPTP (9). These may be mechanisms through which complex I-driven respiration is reduced, i.e., complex I-driven respiration is reduced after the opening of mPTP (22). It is conceivable that reduced complex I subunit transcripts reflect depleted gene products and disrupted function of the long-range, proton-coupled, electron-transfer process (23). To confirm downregulation of complex I after mild expression of APOL1 in G1 and G2 cells, we assessed transcript levels of the N module of complex I core subunits NDUVF2 and NDUFS1 (24), directly responsible for electron-transfer activity to form mitochondrial membrane potential (25). Results were consistent with our previously reported Affymetrix Array data (7). Transcript levels of NDUVF2 and NDUFS1, highly specific N module core subunits of mitochondrial complex I, likely reflect the capacity of electron transport to form the proton electrochemical potential and overall complex I function.

After mitochondrial complex I dysfunction develops, reduced activity of complexes II–IV is expected, with slowing of TCA cycle activity and reduced generation of ATP. Due to the instability of ATP and time lag between cell collection and metabolite measurement, we excluded ATP reads during the initial QC check. However, we performed a luminescent ATP-detection assay to measure ATP levels in HEK293 Tet-on cells immediately after termination of Dox induction (Supplemental Figure 3A). The ATP levels in G1 and G2 cells were significantly reduced after Dox induction (P=2.2×10−5 and P=1.1×10−5, respectively) compared with those without Dox induction, after controlling for the cell numbers per well. After Dox induction, ADP levels were higher in HEK293 Tet-on G1 and G2 cells than G0 cells (P=0.04 and P=0.03, respectively), and AMP levels were higher in G1 than G0 cells (P=0.004) (Supplemental Figure 3B). Because CPT1 levels did not change after Dox induction in either APOL1 G0, G1, or G2 cells (7), we suspect entry of long-chain acyl-CoA into mitochondria is normal. This finding suggests that reductions in acylcarnitines reflect compensation increases in fatty-acid β-oxidation to produce ATP. This process results in greater production of reactive oxygen species (26) and increased demand for
glutathione to counter effects of oxidative stress. The decrease in multiple GG amino acids and cysteine is likely compensatory. Findings were supported by increased levels of methionine sulfone and methionine sulfoxide, markers of oxidative stress. Table cells in green indicate significant differences ($P<0.05$) between groups, metabolite ratio $<1.00$; red indicates significant differences ($P<0.05$) between groups, metabolite ratio $\geq 1.00$; light red trended toward statistical significance ($0.05<P<0.10$), metabolite ratio $\geq 1.00$. EV, empty vector; GGT, $\gamma$-glutamyl transferase; ROS, reactive oxygen species.

This is the first study, of which we are aware, that assessed APOL1 effects on cells on the basis of metabolomics. Because mitochondria are the major intracellular powerhouse, metabolomics is especially useful to analyze mitochondrial pathways. Limitations include that only the top 30 biochemicals were used to explore pathways attributable to expression of APOL1 KRVs (we were unable to investigate unknown metabolites, even those that were highly ranked), and metabolomics may not be optimal to assess all pathologic effects of APOL1; for example, cellular injury resulting from endoplasmic reticulum (ER)/Golgi dysfunction may be less evident due to a lack of signature metabolites (29). Some metabolite changes in KRV-expressing cells were less pronounced in G2 (versus G1) cells. This may indicate a milder phenotype in G2 cells, or could suggest additional and unknown G1- or G2-specific pathways are involved in the development of kidney disease. Our analyses used HEK293 cells. It can be argued that these cells may not fully recapitulate effects in podocytes and renal tubule cells; however, APOL1 KRV-induced mitochondrial fission in HEK293 cells is present in human podocytes with overexpression of APOL1 C-terminal variants (11) and primary proximal tubule cells with increased expression of G1 and G2 (10). Finally, we did not directly measure mitochondrial complex I enzyme activity. Complex I activity assays measure overall NADH dehydrogenase activity; this complicates our approach because NADH dehydrogenase is present on the membranes of mitochondria, ER, Golgi, and plasma membranes (30). Crudely collected mitochondria

Figure 4. | APOL1 kidney risk variants alter redox homeostasis. Mild overexpression of APOL1 G1 and G2 variants increased the levels of methionine sulfone and methionine sulfoxide, markers of oxidative stress. Table cells in green indicate significant differences ($P<0.05$) between groups, metabolite ratio $<1.00$; red indicates significant differences ($P<0.05$) between groups, metabolite ratio $\geq 1.00$; light red trended toward statistical significance ($0.05<P<0.10$), metabolite ratio $\geq 1.00$. EV, empty vector; GGT, $\gamma$-glutamyl transferase; ROS, reactive oxygen species.

<table>
<thead>
<tr>
<th>Sub Pathway</th>
<th>Biochemical Name</th>
<th>G1</th>
<th>G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-glutamyl Amino Acid</td>
<td>gamma-glutamylcysteine</td>
<td>1.07</td>
<td>1.00</td>
</tr>
</tbody>
</table>
from differential centrifugation contain large amounts of mitochondria-associated membranes, primarily ER membranes (31). Proper homogenization and ultracentrifugation are key to obtaining purified mitochondria. However, it is challenging to determine the extent of homogenization. Insufficient homogenization will result in contamination of mitochondria-associated membranes; over-homogenization may produce purified mitochondria but insufficient mitochondria for assay. Instead, transcript levels of NDUFV2 and NDUFS1 were assessed.

Substantial changes in the metabolomic profiles of G0 cells were also detected with Dox induction, particularly a higher level of cysteine (P=0.0002, q=0.004; see Supplemental Table 3). Cysteine is an essential substrate for the primary cellular antioxidant glutathione (GSH) (32,33), and higher cysteine reservoirs protect from oxidative stress (34) and correspond to lower levels of oxidative-stress markers methionine sulfone and methionine sulfoxide (seen in Figure 4C). This supports potential beneficial effects of mild G0 overexpression, in line with increased APOL1 G0 protein countering the effects of BAK1-mediated mitochondrial fission (10).

In addition to critical metabolites, supporting mitochondrial pathways likely affected by APOL1 KRVs, we further inspected transcripts encoding key enzymes mediating impairment in the TCA cycle (i.e., transcripts encoding enzymes between citrate and α-ketoglutarate). After Dox-induced APOL1 expression, AC02 (the mitochondrial form of aconitase) had reduced expression in both G1 and G2 cells, compared with G0 cells (7). We further assessed RNA-sequencing data from the HEK293 Tet-on cell model. The AC02 transcript was significantly reduced in G1 cells compared with G0 cells, and IDH2 (the mitochondrial form of isocitrate dehydrogenase) was significantly reduced in both G1 and G2 cells compared with G0 cells (Supplemental Figure 4). These data provide additional evidence that reduction in complex I (type-I NADH dehydrogenase) played a key role in limiting the conversion of α-ketoglutarate to α-ketoglutarate. Accumulation of citrate is expected. It is likely the outcome of reduced demand for conversion of citrate to α-ketoglutarate by ACO2, and reduced demand for IDH2 catalyzing the conversion of isocitrate to α-ketoglutarate (due to the lack of NAD⁺ as a mitochondrial coenzyme for α-ketoglutarate dehydrogenase to convert α-ketoglutarate to succinyl-CoA). AC02 and IDH2 are coexpressed, and complex NDUFB10 and NDUFS1 units are considered “elite partners” of AC02 (genecards.org) in addition to

Figure 5. | APOL1 kidney risk variants alter one-carbon metabolism and transsulfuration. Several intermediates in this pathway were increased in G1-expressing cells, including methionine, S-adenosyl methionine (SAM), S-adenosylhomocysteine (SAH), cystathionine, and taurine; whereas cysteine was reduced in G1- and G2-expressing cells. Results demonstrate differential effect of G1 and G2 variants. CH₃, methyl group; EV, empty vector; GCS, glutamylcysteine synthetase; GS, glutathione synthetase.

| EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) | EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) | EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) |
| EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) | EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) | EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) |
| EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) | EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) | EV(-) G0(-) G0(+)G1(-) G1(+)G2(-) G2(+)EV(+) |
coexpression with ACO2 and IDH2. Both NDUF10 and NDUF51 transcripts were captured in the pattern analysis as having reduced expression in G1 and G2 cells, compared with G0 cells (7).

It is noteworthy that APOL1 G1 and G2 variants increase the risk of kidney disease and more rapid failure of kidney allografts, without effects on other organs (35). A future investigation of plasma metabolomics in healthy individuals of African descent with differing APOL1 genotypes will help clarify whether APOL1 KRVs affect general metabolic features in vivo.

In conclusion, biochemicals identified in a metabolomics analysis of cells expressing APOL1 revealed a number of altered pathways attributable to G1 and G2 KRVs. Changes appear likely to result from collapse of the mitochondrial membrane potential induced by mitochondrial fragmentation/fission (10) and/or opening of mPTP (9). This would cause cellular dysfunction due to alterations in the TCA cycle, increased fatty-acid oxidation, and unbalanced redox homeostasis. This integrated pattern of effects support that complex I deficiency induced by mitochondrial membrane potential depletion (22) is the primary cause of APOL1-associated kidney disease, increased fatty-acid oxidation is needed to compensate for TCA-cycle deficiency, and unbalanced redox homeostasis is a consequence of excessive β-oxidation. Future studies will focus on crossplatform analyses, including metabolites and enzymes that catalyze key substrates in metabolic networks. A systematic pairwise analysis may identify APOL1 G1- or G2-specific effects causing pathologic outcomes beyond mitochondrial metabolic pathways.

Disclosures
Wake Forest University Health Sciences and B. Freedman have rights to an issued US patent related to APOL1 genetic testing. B. Freedman is a consultant for AstraZeneca Pharmaceuticals and RenalytixAI. All remaining authors have nothing to disclose.

Funding
This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grants R01 DK070941 and R01 DK084149 (to B. Freedman). J. Snipes reports receiving grants from Wake Forest Health Sciences, during the conduct of the study.

Acknowledgments
We thank Dr. Martin Pollak for sharing APOL1 vectors, and Dr. Gregory R. Wagner and Dr. Gregory A. Michelotti for sample processing and retrieval of metabolomics data.

Author Contributions
Y. Choi, B. Freedman, and L. Ma were responsible for investigation; B. Freedman and L. Ma conceptualized the study, were
responsible for funding acquisition, and wrote the original draft; B. Freedman, L. Ma, and N. Palmer were responsible for resources; B. Freedman, L. Ma, and J. Parks were responsible for supervision; B. Freedman, M. Murea, N. Palmer, and J. Snipes were responsible for project administration; C. Langefeld and L. Ma were responsible for formal analysis; L. Ma was responsible for data curation; L. Ma, M. Murea, J. Parks, and J. Snipes were responsible for methodology; and all authors reviewed, edited, and approved the manuscript.

Supplemental Material
This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl?doi=10.34067/KID.202002000359/DCSupplemental.

Supplemental Figure 1. Outline of experimental groups of HEK293 Tet-on cell lines.

Supplemental Figure 2. APOL1 KRVs alter the TCA cycle.

Supplemental Figure 3. APOL1 kidney risk variants cause energy depletion.

Supplemental Figure 4. APOL1 kidney risk variants reduced the transcription levels of AC2O and IDH2.

Supplemental Table 1. Metabolite reads (original scale).

Supplemental Table 2. Metabolite reads (normalized).

Supplemental Table 3. Significance tests for biochemicals in the current metabolomics analysis.

References


24. Tosca MG, Vazquez EJ, Chen Q, Kerner J, Kern TS, Hoppel CL: Oxidation of fatty acids is the source of increased mitochondrial


Received: June 2, 2020 Accepted: September 29, 2020
Supplemental Figure 1. Outline of experimental groups of HEK293 Tet-on cell lines

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Genotype</th>
<th>Genotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Empty Vector</td>
<td>APOL1</td>
<td>APOL1</td>
<td>APOL1</td>
</tr>
<tr>
<td></td>
<td>EV</td>
<td>G0</td>
<td>G1</td>
<td>G2</td>
</tr>
<tr>
<td>Dox (-)</td>
<td>N=3</td>
<td>N=3</td>
<td>N=3</td>
<td>N=3</td>
</tr>
<tr>
<td>Dox (+)</td>
<td>N=3</td>
<td>N=3</td>
<td>N=3</td>
<td>N=3</td>
</tr>
</tbody>
</table>

*Cells were grown in 150 mm cell culture dishes. Experiments were done in triplicate.*
Supplemental Figure 2. *APOL1* KRVs alter the TCA cycle. Mild overexpression of *APOL1* G1 and G2 variants altered the pattern of metabolites in the TCA cycle. The altered pattern of metabolites matched deficiency of type I NADH dehydrogenase or mitochondrial complex I. Red arrows refer to increased metabolites and green arrows reflect reduced metabolites, corresponding to those in Figure 1.

The TCA cycle backbone image is adopted from https://employees.csbsju.edu/cschaller/Reactivity/tcacycle/TCAoverview.html
### Biochemical Name

<table>
<thead>
<tr>
<th></th>
<th>Fold of Change (with DOX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( G_1 )</td>
</tr>
<tr>
<td>adenosine 5(^{'})-diphosphate (ADP)</td>
<td>1.73</td>
</tr>
<tr>
<td>adenosine 5(^{'})-monophosphate (AMP)</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Supplemental Figure 3

\( p = 2.2 \times 10^{-5} \)

\( p = 1.1 \times 10^{-5} \)
Supplemental Figure 3. *APOL1* kidney risk variants cause energy depletion. Mild overexpression of *APOL1* G1 and G2 variants a) reduced intracellular ATP content measured by a luminescent ATP detection assay (Abcam, Cat # ab113849) after controlling for the total number of cells in each well (10,000 cells/well when seeded) on a lumox 96-well plate (Sarstedt, Germany), using a BioTek Synergy HTX multi-mode plate reader (BioTek, Winooski, VT); and b) increased ADP levels in G1 and G2 cells and increased AMP levels in G1 cells. Red indicates significant between-group differences (p≤0.05), metabolite ratio ≥1.00 (original data from Supplementary Table 3).
Supplemental Figure 4
Supplemental Figure 4. *APOL1* kidney risk variants reduced the transcript levels of *ACO2* and *IDH2*. Mild overexpression of *APOL1* G1 and G2 variants a) reduced *ACO2* (the mitochondrial form of aconitase) by RNA sequencing; b) reduced *IDH2* (the mitochondrial form of isocitrate dehydrogenase) via RNA sequencing.