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ORIGINAL ARTICLE

A null variant in the *apolipoprotein L3* gene is associated with non-diabetic nephropathy

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ABSTRACT

Background. Inheritance of apolipoprotein L1 gene (*APOL1*) renal-risk variants in a recessive pattern strongly associates with non-diabetic end-stage kidney disease (ESKD). Further evidence supports risk modifiers in *APOL1*-associated nephropathy; some studies demonstrate that heterozygotes possess excess risk for ESKD or show earlier age at ESKD, relative to those with zero risk alleles. Nearby loci are also associated with ESKD in non-African Americans.

Methods. We assessed the role of the *APOL3* null allele rs11089781 on risk of non-diabetic ESKD. Four cohorts containing 2781 ESKD cases and 2474 controls were analyzed.

Results. Stratifying by *APOL1* risk genotype (recessive) and adjusting for African ancestry identified a significant additive

association between rs11089781 and ESKD in each stratum and in a meta-analysis [meta-analysis P = 0.0070; odds ratio (OR) = 1.29]; ORs were consistent across *APOL1* risk strata. The biological significance of this association is supported by the finding that the *APOL3* gene is co-regulated with *APOL1*, and that APOL3 protein was able to bind to APOL1 protein.

Conclusions. Taken together, the genetic and biological data support the concept that other APOL proteins besides APOL1 may also influence the risk of non-diabetic ESKD.

Keywords: African American, APOL1, APOL3, chronic kidney disease, FSGS

INTRODUCTION

The G1 and G2 coding variants in the apolipoprotein L1 gene (*APOL1*) on chromosome 22q13 are associated with a spectrum of non-diabetic etiologies of chronic kidney disease (CKD) [1–4]. This observation altered our understanding of the pathogenesis of nephropathy in populations with recent African ancestry. The biology of the *APOL1* gene product and the mechanisms whereby high-risk *APOL1* variants cause kidney disease remain incompletely elucidated. Initial structural characterization of the C-terminal coiled coil domains of G0 (wild-type), G1 and G2 APOL1 proteins has recently been reported [5]. In addition, *APOL1* kidney disease risk variant cytotoxicity is accompanied by mitochondrial dysfunction [6], as well as cellular potassium depletion with subsequent induction of stress-activated protein kinases [7].

APOL1-associated nephropathy risk is inherited in an autosomal recessive pattern, such that risk of kidney disease is greatly increased in individuals possessing two copies of the G1 and/or the G2 nephropathy variants [1]. Limou *et al.* proposed a multimer model for the cellular toxicity of APOL1 protein in order to reconcile toxic gain-of-function mutations with autosomal recessive inheritance [8]. Nonetheless, individuals with a single *APOL1* nephropathy variant may have small but significant increases in disease risk and initiate renal replacement therapy at earlier ages than do those without *APOL1* nephropathy variants [1, 9–11]. Moreover, most African Americans who inherit two *APOL1* nephropathy variants do not develop CKD [1, 12, 13].

Factors in addition to *APOL1* G1 and G2 genotypes appear to modulate risk for *APOL1*-associated kidney disease. Variation at genetic loci near *APOL1* may contribute, a hypothesis supported by the association of markers in neighboring genes with CKD in European and Asian ancestry populations [14–17]. Association between end-stage kidney disease (ESKD) and an *APOL3* null variant (nonsense mutation rs11089781) was initially described by Tzur *et al.* in African Americans from New York [2]. Subsequently, Next Generation DNA sequencing in African Americans from the southeastern USA revealed an interaction between *APOL1* and *APOL3* rs11089781 in individuals with fewer than two *APOL1* G1/G2 renal-risk alleles [18]. This association was not statistically significant in African American participants in the Family Investigation of Nephropathy and Diabetes (FIND) study, though the odds ratio (OR) was consistent in direction. To gain a more complete understanding of the complex genetics involved in the contribution of the *APOL3* gene to kidney disease, additional cohorts of non-diabetic ESKD cases and controls with *APOL1* and *APOL3* genotype data were evaluated. Cell-based, gene regulation and protein co-localization studies were also performed in order to gain further insight into the underlying biological mechanisms.

MATERIALS AND METHODS

Study populations

Four study sample sets were analyzed and included in the meta-analysis. FIND participants included 807 African American cases with non-diabetic etiologies of ESKD [HIV-associated nephropathy (HIVAN), focal segmental glomerulosclerosis (FSGS), primary or systemic forms of chronic glomerulonephritis, hypertension-attributed and other etiologies] and 634 African American controls [18, 19]. Wake Forest participants included 1559 African American cases with non-diabetic ESKD attributed to FSGS, focal global glomerulosclerosis, HIVAN, chronic glomerulonephritis without a kidney biopsy, hypertension or unknown causes, along with 1332 controls with a serum creatinine <1.5 mg/dL (men) or <1.3 mg/dL (women), and (when available) urine albumin:creatinine ratio <30 mg/g [18]. New York Hispanic Americans admixed with recent African ancestry included participants whose families came primarily from the Dominican Republic and Puerto Rico (83 non-diabetic ESKD cases, 365 non-nephropathy controls) [2, 20]. New York African Americans included 332 non-diabetic ESKD cases and 143 controls [2, 20]. Cases with ESKD in both New York ethnic groups included patients with HIVAN, non-monogenic forms of FSGS and kidney disease attributed to hypertension in the absence of other known causes.

All four sample sets were genotyped for nephropathy risk alleles *APOL1* G1 (rs73885319; rs60910145) and *APOL1* G2 (rs71785313), for *APOL3* null allele rs11089781 (Gln58Ter), and for ancestry-informative markers to estimate proportion of African ancestry. Methods and quality control from each study have been reported [1, 2, 18–20].

Statistical analyses

To test for an association of the *APOL3* null mutation rs11089781 with ESKD status, a logistic regression model was computed, stratified by *APOL1* renal risk genotype status and adjusted for global percentage African ancestry as a covariate. Specifically, the model was stratified into two groups, with the first stratum defined as individuals with 0 or 1 copy of the G1 or G2 *APOL1* risk alleles and the second stratum defined as individuals with 2 copies of the G1 and/or G2 *APOL1* risk alleles. The effect of rs11089781 was analyzed in an additive model, in which risk increases with the number of *APOL3* stop codon variants A. The prevalence of the 2 stop codon (variant A) genotype was extremely low in New York Hispanic samples (only four homozygotes), limiting our ability to analyze a recessive model. The logistic regression models were computed separately in each of the four data sets, as the sample characteristics were different. To test the hypothesis of association across cohorts, inverse normal meta-analyses were computed [21] without or with weighting by the square root of sample size.

The corresponding weighted and unweighted OR and 95% confidence intervals (95% CIs) were computed. Finally, the two *APOL1* risk strata were combined via the weighted and unweighted inverse normal meta-analysis method. A two-sided P-value is reported.

Cells and reagents

Primary human coronary arterial endothelial cells (HCAEC) were purchased from Lonza (Basel, Switzerland), and experiments were performed at passage 8. HCAEC were cultured in EGM-2 media with MV Bulletkit/5% FBS (Lonza). SV40 T antigen and hTERT virus-immortalized human podocytes were cultured in RPMI/10%FBS/pen-strep/ITS (kind gift of Dr Jeffrey Kopp). Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza, cultured in EGM-2 with Bulletkit/2% FBS (Lonza), and experiments were performed on cells at passage 5–6. Human embryonic kidney (HEK293) cells were purchased from ATCC (Manassas, VA, USA) and cultured in Eagle's minimal essential media with 10% FBS and pen/strep mix. Interferons were purchased from PBL Interferon Source (Piscataway, NJ, USA) or eBioscience (San Diego, CA, USA).

mRNA expression

mRNA was isolated from cells using the RNeasy kit (Qiagen, Venlo, The Netherlands), reverse transcribed into cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems, Grand Island, NY, USA), and quantified by real-time PCR using an Applied Biosystems 7500 PCR system (Grand Island, NY, USA). Expression of the mRNA expression was normalized to either 18S subunit or β-actin expression. Real-time primer-probes from Applied Biosystems were: APOL1 Hs00358603_g1, 18S 4319413E, β-Actin 4326315E.

Bioinformatic studies

APOL expression data in lymphoblasts was obtained from Stranger *et al.* [22] and APOL expression data in human glomeruli was obtained from Nephroseq (www.nephroseq.org). Calculations were performed using log2 transformed data.

Co-transfection and lysate collection

HEK293T cells were grown to 70–80% confluence prior to transfection with *APOL1* and C-terminal myc-FLAG-tagged *APOL3* plasmids (Origene RC220066; Rockville, MD, USA). Co-transfection was performed using Lipofectamine® 2000 and 20 μg of each plasmid, according to the manufacturer's protocol (Life Technologies #11668027, Grand Island, NY, USA). Opti-MEM® media was brought to a final concentration of 10% FBS at 4 h post-transfection. Cells were lyzed and collected in Brij99 detergent 24 h post-transfection.

Co-immunoprecipitation

Lysate samples containing 750 μg of protein were pre-cleared in a 1:1 mixture of Protein A and Protein G Dynabeads® (Life Technologies 10001D & 10003D). Pre-cleared supernatant was isolated and incubated at 4°C overnight with 3 μg of the immunoprecipitation (IP) antibody [anti-DDK Origene TA500011 or anti-APOL1 Sigma Aldrich HPA018885 (St Louis, MO, USA)]. Samples were incubated with DynaBeads® for 1 h rotating at 4°C to bind the IP antibody, and beads were washed 5× with Brij99 buffer prior to eluting the antibody and its accompanying proteins off of the beads in lithium dodecyl sulfate (LDS) non-reducing buffer (Thermo Scientific #84788, Pittsburgh, PA, USA) at 70°C for 10 min. The vector backbone for the *APOL1* clone was pCMV-AN-HA and the vector for *APOL3* was pCMV6-Entry (Origene). The *APOL1* sequence was generated to match NCBI Reference Sequence NP_001130013.1; genotypes were altered with site-directed mutagenesis. All clones were verified by sequencing.

Western blot detection

IP eluates and lysate control samples were run on denaturing Tris-HCl precast gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in non-fat milk and then incubated in primary antibody overnight at 4°C followed by washing and incubation in secondary antibody for 1 h at room temperature. Proteins were visualized using the SuperSignal West Pico kit (Pierce/ThermoScientific, Pittsburgh, PA, USA). The same antibodies served for immunoblotting and for immunoprecipitation.

Mass spectroscopy

HUVEC cells with either the *APOL1* G0/G0 or G1/G1 genotype were stimulated with 10 ng/mL of interferon gamma. After 24 h, the cells were lyzed in Brij99 detergent (1% Brij 99, 20 mM Hepes, 50 mM NaCl, 5 mM EDTA). The lysate was pre-cleared with protein A/G beads, then immunoprecipitation was performed with 3 μg of anti-APOL1 antibody (Sigma). Beads were then washed five times in the Brij99 buffer. Protein was separated by gel electrophoresis and APOL1 pull-down verified by colloidal Coomassie staining. Gel sections were excised and sent to the Harvard University core facility for mass spectroscopy (liquid chromatography-mass spectrometry; LC-MS/MS) using a LTQ Orbitrap Elite (ThermoFisher, Waltham, MA, USA) equipped with a Waters NanoAcuity HPLC system. Assignment of MS/MS spectra was performed using the Sequest algorithm to search the Human Uniprot Database (SwissProt 2014). Data was analyzed using in-house Proteome Browser Suite (PBS) software.

Yeast two-hybrid interactions

DNA constructs coding for amino acids 238–398 (either G0, G1 or G2) were cloned into the pB27 bait vector with the LexA DNA-binding domain upstream (N-terminal) to the APOL1 bait sequence. The prey sequences were derived from a randomly primed human placenta cDNA library for maximum transcript representation. Interaction of bait with prey proteins allowed LexA transcription of His3 (necessary for histidine synthesis) and yeast growth on media lacking histidine. Yeast colonies were sequenced across the prey insert sites to identify the protein fragments encoded.

Immunostaining

HEK293 cells on collagen-coated coverslips were co-transfected with hemagglutinin-tagged APOL1 (transcript variant 4) and FLAG-tagged APOL3 using Lipofectamine 3000.

Table 1. Association of *APOL3* single-nucleotide polymorphism rs11089781 with ESKD in four cohorts, stratified by *APOL1* risk genotype status and adjusted for admixture proportions

Sample	ESKD	Number	Allele frequency	Odds ratio	95% CI	P-value
***APOL1* low-risk genotype group (0 or 1 copy of renal-risk variants)**[a]						
Wake Forest AA	Cases	837	0.21	1.12	0.95–1.31	0.1905
	Controls	1136	0.19			
FIND AA	Cases	430	0.21	1.02	0.81–1.28	0.8709
	Controls	561	0.20			
New York AA	Cases	199	0.19	1.62	1.02–2.56	0.0410
	Controls	126	0.12			
New York HA	Cases	65	0.10	2.10	1.04–4.22	0.0380
	Controls	354	0.05			
Unweighted meta-analysis	Cases	1531	0.18	1.40	1.12–1.75	0.0029
	Controls	2177	0.14			
Weighted[b] meta-analysis	Cases	1531	0.20	1.29	1.09–1.53	0.0034
	Controls	2177	0.17			
***APOL1* high-risk genotype (2 copies of renal-risk variants)**[c]						
Wake Forest AA	Cases	722	0.40	1.03	0.82–1.29	0.8056
	Controls	196	0.39			
FIND AA	Cases	377	0.40	1.20	0.83–1.75	0.3366
	Controls	73	0.36			
New York AA	Cases	133	0.38	1.30	0.61–2.76	0.4980
	Controls	17	0.32			
New York HA	Cases	18	0.47	3.44	0.80–14.79	0.9650
	Controls	11	0.27			
Unweighted meta-analysis	Cases	1250	0.41	1.53	1.00–2.34	0.0486
	Control	297	0.34			
Weighted[d] meta-analysis	Cases	1250	0.40	1.24	0.98–1.56	0.0740
	Controls	297	0.37			

AA, African American; HA, Hispanic American.
[a]No evidence of heterogeneity of the odds ratio for unweighted (P-value = 0.11) or weighted (P-value = 0.19) analyses.
[b]Weighted analysis weights by the square root of the sample size of the cohort with 0 or 1 copy of *APOL1* renal-risk alleles.
[c]No evidence of heterogeneity of the odds ratio for unweighted (P-value = 0.38) or weighted (P-value = 0.90) analyses.
[d]Weighted analysis weights by the square root of the sample size of the cohort with 2 copies of *APOL1* renal-risk alleles.

After 16 h, cells were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100, and blocked with goat serum. Cells were probed with mouse anti-FLAG antibody (Sigma) and rabbit anti-HA antibody (Sigma). Secondary antibodies were goat anti-mouse Alexa Fluor 488 Green (ThermoFisher) and goat anti-rabbit Alexa Fluor 555 Red (ThermoFisher). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were imaged on a Laser Scanning Microscope (LSM) 880 Confocal Laser System.

RESULTS

Genetic analysis

Table 1 summarizes the *APOL3* null variant rs11089781 associations with ESKD in four case–control samples enriched for non-diabetic ESKD: FIND non-diabetic African Americans, Wake Forest African Americans, New York African Americans and New York Hispanic Americans. The New York Hispanic American sample originated largely from the Dominican Republic and Puerto Rico, but contained recent African ancestry due to admixture with African Americans [2].

The *APOL3* rs11089781 A allele (null allele) was present at higher frequencies in all four ESKD case sets, compared with their respective controls. Global African ancestry proportion approximated 80% in FIND, Wake Forest and New York African Americans, in contrast to 34% and 31%, respectively, in New York Hispanic American ESKD cases and controls. ORs and 95% CIs for the additive effect of the *APOL3* rs11089781 stop codon on risk for non-diabetic ESKD, accounting for global ancestry, are reported, stratified by *APOL1* risk genotype (two renal-risk alleles, less than two renal-risk alleles) and by cohort. Meta-analyses using the unweighted and sample size-weighted inverse normal method showed a statistically significant additive effect in those with two *APOL1* renal-risk alleles and in those with less than two *APOL1* renal-risk alleles (Table 1). The OR was >1.0 in all cohorts and across both *APOL1* risk genotype strata, showing an increased risk based on the number of copies of the minor allele.

Given the consistent pattern of risk across *APOL1* risk genotype strata, a combined analysis was computed that adjusted for admixture and *APOL1* risk genotype (Table 2). Here, both the weighted and unweighted analyses showed a strong statistical association between rs11089781 and ESKD ($OR_{unweighted}$ = 1.43, $P_{unweighted}$ = 0.00027; $OR_{weighted}$ = 1.29, $P_{weighted}$ = 0.0070). Of note, the magnitude of the effect (OR = 2.34) and statistical significance (P = 0.0066) was greatest in the New York Hispanic sample, generalizing results beyond individuals with high proportions of recent African ancestry.

Gene regulation of *APOL1*, *APOL2* and *APOL3*

Bioinformatic analysis of *APOL*-region gene expression was performed using HapMap lymphoblast data. Results showed very

Table 2. Association of *APOL3* single-nucleotide polymorphism rs11089781 with ESKD in four cohorts, adjusting for *APOL1* risk genotype status and admixture proportions

Sample	ESKD	*n*	Allele frequency	Odds ratio	95% CI	P-value[a]
Wake Forest AA	Cases	1559	0.30	1.09	0.95–1.24	0.2171
	Controls	1332	0.22			
FIND AA	Cases	807	0.30	1.07	0.88–1.30	0.4940
	Controls	634	0.22			
New York AA	Cases	332	0.27	1.52	1.03–2.26	0.0362
	Controls	143	0.15			
New York HA	Cases	83	0.18	2.34	1.27–4.33	0.0066
	Controls	365	0.06			
Unweighted meta-analysis	Cases	2781	0.26	1.43	1.18–1.72	0.00027
	Control	2474	0.16			
Weighted[b] meta-analysis	Cases	2781	0.28	1.29	1.12–1.48	0.00701
	Controls	2474	0.20			

AA, African American; HA, Hispanic American.
[a]No evidence of heterogeneity of the odds ratio for unweighted (P-value = 0.11) or weighted (P-value = 0.09) analyses.
[b]Weighted analysis weights by the square root of the sample size of the cohort.

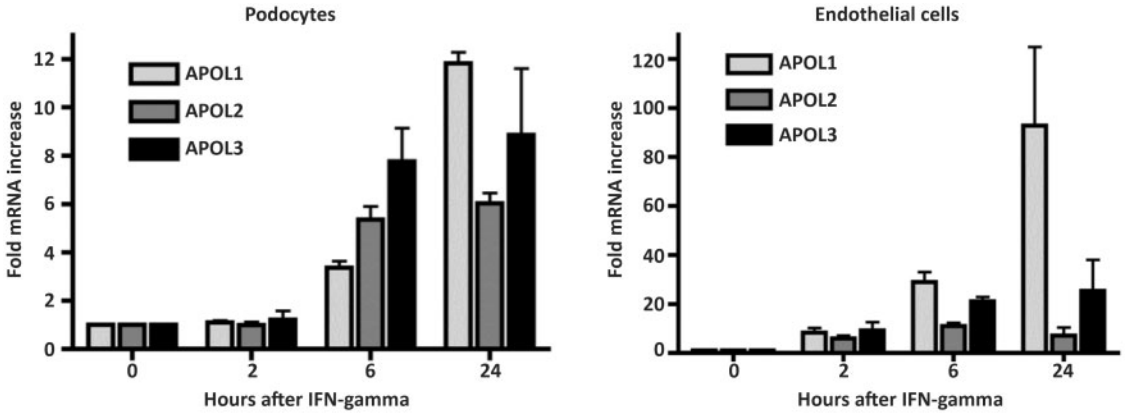

FIGURE 1: Co-regulation of *APOL* genes by interferon. (Left) Podocytes or (right) endothelial cells were treated with 1 ng/mL IFNγ. *APOL* gene expression was measured 2, 6 or 24 h after interferon stimulation. *APOL1*, *APOL2* and *APOL3* were all upregulated by IFNγ in both cell types. In endothelial cells short-term exposure to IFNγ upregulated *APOL* mRNAs to similar degrees, but *APOL1* expression predominated after prolonged IFNγ exposure.

high correlation between *APOL1* and *APOL2* transcript levels ($R^2 = 0.8$, Supplementary data, Figure S1) and more limited correlation of either gene with *APOL3* levels ($R^2 = 0.07$–0.11) under baseline conditions. *APOL1* transcription is highly responsive to interferon stimulation, and *APOL2* and *APOL3* promoter analysis predicted a similar response. Specifically, unbiased genome-wide chromatin immunoprecipitation assays from the ENCODE project [23] show interferon-induced signal transducer and activator of transcription 1 (STAT1) and STAT2 binding in the regulatory regions of *APOL1*, *APOL2* and *APOL3*, suggesting the possibility of coordinated gene regulation.

To test this regulation, HCAEC and podocytes were treated with interferon-gamma (IFNγ), and mRNA transcript levels from all three genes were measured (Figure 1). At early time points, expression of the three *APOL* genes increased to similar degrees, but prolonged interferon exposure resulted in sustained *APOL1* expression relative to *APOL2* and *APOL3*. This response suggests that a high ratio of *APOL1* to *APOL2* or *APOL3* may be a signature of the high interferon state.

In order to validate the coordinated regulation of these *APOL* genes in human kidneys, we used Nephroseq gene expression data from isolated glomeruli to analyze the correlation between *APOL* transcript levels in health and disease (Table 3). In glomeruli from normal kidneys, *APOL1* and *APOL2* expression showed only minimal correlation, whereas neither was correlated with *APOL3*. However, in glomeruli from kidneys with a variety of disease states, expression levels of these APOL genes exhibited stronger correlations. These data strengthen the hypothesis that expression of *APOL2* and *APOL3* is modulated in concert with changes in *APOL1* expression, suggesting a coordinated regulatory region with important functional implications.

Physical interaction of *APOL1* with *APOL3*

In parallel experiments, a yeast two-hybrid screen was performed to identify *APOL1* binding partners using as bait the C-terminal aa 238–398 of *APOL1*. *APOL3* was identified as an *APOL1* binding partner, with the *APOL1* C-terminus binding to a polypeptide fragment of *APOL3* (*APOL3* isoform 1 aa 100–287; Supplementary data, Figure S2). To confirm this interaction, *APOL1* and *APOL3*-FLAG expression plasmids were transfected into HEK293 cells. Different splice variant isoforms

of the *APOL1* gene product alter the signal peptide and its cleavage and are thus expected to have different intracellular localization and secretory pathway patterns [24]. Accordingly, we used an *APOL1* transcript variant (tv)4 lacking a signal peptide in order to express both proteins in the same cellular compartments. *APOL1* pull-down co-immunoprecipitated *APOL3*-FLAG and, conversely, pull-down of *APOL3*-FLAG co-immunoprecipitated *APOL1* (Figure 2). No apparent differences were observed in *APOL3* affinity among *APOL1* polypeptides of different risk genotypes [G0 (wild-type), G1 and G2].

Potential interactions were tested under more physiologic conditions by performing immunoprecipitations in IFNγ-treated endothelial cells. In the absence of antibodies that reliably and specifically bind native *APOL3*, *APOL1* was pulled down with anti-*APOL1* antibody and mass spectroscopy was used to detect *APOL3* (Table 4). Endothelial cells of *APOL1* G0/G0 and G1/G1 genotypes were tested (we were unable to obtain endothelial cells with the less common G2/G2 genotype). Pull-down fractions of both G0 and G1 variants of *APOL1* contained peptide spectra unique to *APOL3*. Thus, a physical interaction was supported in three different experimental systems: yeast two-hybrid, plasmid-transfected HEK cells and IFNγ- induced primary endothelial cells.

Table 3. Correlation between *APOL1*, *APOL2* and *APOL3* gene expression in humans

Kidney disorder	Number	*APOL1/APOL2*	*APOL1/APOL3*	*APOL2/APOL3*
Normal	41	0.10	0.01	0.02
FSGS	25	0.30	0.20	0.45
Lupus nephritis	32	0.46	0.31	0.64
IgA nephropathy	27	0.61	0.12	0.44
Membranous nephropathy	21	0.46	0.11	0.14
Minimal change disease	13	0.54	0.09	0.47
Diabetic nephropathy	12	0.56	0.40	0.71
RPGN	23	0.33	0.26	0.34

RPGN, rapidly progressive glomerulonephritis. Human glomerular gene expression profiling data from Nephroseq were used to compare APOL correlate transcript abundance between *APOL* genes. In kidneys from normal donors, expression correlation is low, whereas disease states drive coordinated regulation. Correlation is shown as the correlation coefficient R^2.

To begin probing the nature of the *APOL1*/*APOL3* interaction, we tested whether altering *APOL3* levels influenced *APOL1*-mediated cytotoxicity. We were unable either to rescue *APOL1* toxicity in HEK293 cells by co-transfection of *APOL3* or to exacerbate *APOL1* toxicity in interferon-stimulated endothelial cells via siRNA knockdown of *APOL3* (data not shown), possibly due to the need for other upregulated proteins in a larger complex. We performed immunostaining on HEK293 cells co-transfected with hemagglutinin-tagged *APOL1* and FLAG-tagged *APOL3* in order to identify potential sites of interaction within the cell (Supplementary data, Figure S3). While much of *APOL3* trafficked to a perinuclear aggresome-like organelle, both *APOL1* and *APOL3* are widely distributed throughout the cell and demonstrate broad areas of physical overlap, supporting an interaction taking place in the cell rather than following lysis.

DISCUSSION

We report the detection of a genetic association between *APOL3* null variant rs11089781 and non-diabetic ESKD in populations with recent African ancestry. This finding was present regardless of *APOL1* risk genotype status and degree of African ancestry, and was found in both African Americans and Hispanic Americans with recent African ancestry from New York. Subsequent experiments revealed physical interaction between *APOL1* and *APOL3* proteins in kidney cells. Multimerization of APOL proteins influencing nephropathy susceptibility has been proposed [8]. Although *APOL3* genetic association may be important in explaining the weaker autosomal dominant inheritance of *APOL1* G1 and G2 risk alleles in some reports (compared with the major autosomal recessive inheritance) and the earlier age at ESKD in subjects who possess a single *APOL1* G1 or G2 risk allele, the current study also supports an independent effect of this allele, including in individuals with two *APOL1* renal-risk alleles.

While the genetic association may assist in refining risk prediction, perhaps its most important implication is for understanding potential biological mechanisms of *APOL1*-associated kidney disease. Several reports demonstrate enhanced toxicity

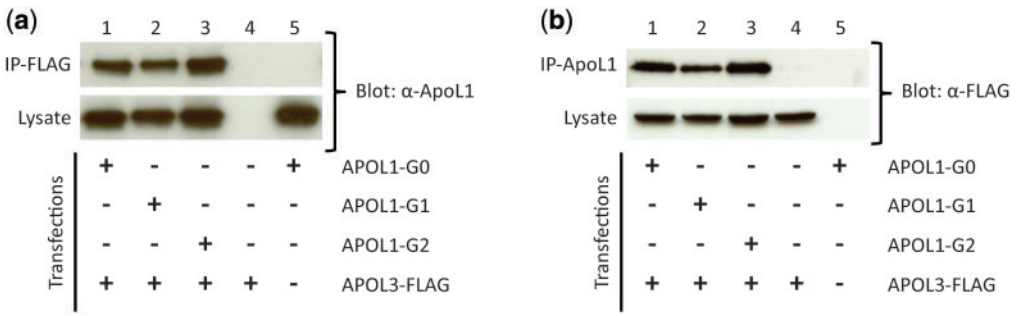

FIGURE 2: Physical interaction between *APOL1* and *APOL3* proteins. (**a**, **b**) Co-transfection of *APOL1* and flag-tagged *APOL3* into HEK293 cells was followed by immunoprecipitation and Western blotting. (a) Pull-down performed on lysates using anti-FLAG antibody (*APOL3*-FLAG) followed by Western blotting with α-*APOL1* antibody (Sigma rabbit polyclonal) demonstrated that *APOL3* binds G0, G1 and G2 *APOL1*. (b) The same lysates and antibodies were used for pull-downs with anti-*APOL1* antibody followed by Western blotting with anti-FLAG antibody. *APOL1* G0, G1 and G2 all pulled down FLAG-tagged *APOL3*.

of the *APOL1* G1 and G2 risk alleles, relative to the G0 wild-type *APOL1* allele [25–27]. However, these reports show that wild-type *APOL1* may not be entirely benign with respect to tissue injury. The small excess risk of disease in *APOL1* risk allele heterozygotes from genetic studies is also consistent with gain-of-function toxicity of risk variants. *APOL1* protects humans and a few other primate species from pathogens, while *APOL1* risk variants appear to protect against a wider range of pathogens, explaining the rapid rise in *APOL1* renal-risk variant allele frequency in Africa [1, 27]. However, this protection against an extended spectrum of pathogens may come at the expense of greater propensity to *APOL1*-mediated tissue injury. We propose that co-expression of other members of the *APOL* gene family may mitigate the toxic effects of *APOL1*, those of G0 and of both risk variants [2, 18]. Complete absence of the full-length isoform 1 of *APOL3* may render individuals more vulnerable to *APOL1*-mediated tissue injury (the *APOL3* Q58ter variant theoretically allows possible translation of apparently minor *APOL3* transcript isoforms 2 and 3), but even functional *APOL3* alleles may offer only limited protection when both *APOL1* alleles carry G1 or G2 renal-risk variants. The present analyses strengthen the formulation of possible multimerization of APOL proteins in kidney cells, as an explanation for the risk inheritance mode [7]. On the other hand, since we did not observe rescue of *APOL1*-induced toxicity by *APOL3* co-transfection, it is possible that additional proteins are necessary for *APOL3*-based protection as part of a larger complex, possibly involving one or more of the many other genes that respond to interferon. Another possibility is that *APOL3* acts through other mechanisms to increase the risk of CKD and the genetic interaction observed might be attributed to additive effects in an already sensitized population.

Several kidney diseases associated with *APOL1* risk alleles, such as HIVAN, FSGS and lupus nephritis, are high interferon states [26]. In fact, interferon itself appears to promote kidney disease in individuals with two *APOL1* risk alleles [26]. The data herein show that IFNγ coordinately upregulates the transcription of *APOL1*, *APOL2* and *APOL3*. This suggests that these genes operate as a functional cassette in response to stimuli characteristic of pathogen attack. Direct physical interaction of APOL1 with APOL3 protein within transfected HEK cells and interferon-stimulated endothelial cells provides additional evidence for functional coordination. Mapping of the interaction interfaces and mechanism may be helpful in future efforts to design therapies which attenuate *APOL1*-mediated human disease.

Similar to the *APOL1* G1 variant, the *APOL3* nonsense variant is found only in individuals with recent African ancestry. Although the *APOL3* nonsense allele is physically quite distant from the *APOL1* risk alleles, it is possible that the *APOL3* nonsense allele was carried to high frequency by the same selective sweep that drove the G1 allele to high frequency in Africa [1]. Subsequent recombination appears to have weakened linkage disequilibrium between these loci ($D' = 0.57$; $R^2 = 0.12$). However, the *APOL3* null allele may have been co-selected with G1, perhaps because G1 may be more efficient in pathogen protection when not bound by *APOL3*. Evolutionary forces would have governed the balance between such pathogen protection on one hand, and human cell and tissue injury on the other. Further functional studies will be needed to understand the full ramifications of the biological interactions among *APOL* family members. The results of such studies have the potential to inform treatment strategies directed against *APOL1*-mediated nephrotoxicity.

In conclusion, human genetic and functional experiments support a modulatory role of the *APOL3* gene in nephropathy susceptibility. We postulate that in the presence of a functional *APOL3* gene and abundant APOL3 protein, *APOL1* G1 or G2 alleles may be less likely to cause kidney disease.

Table 4. Peptide spectra uniquely mapping to *APOL3* with *APOL1* pull-down

Unique APOL3 peptide spectra	
APOL1 WT allele	APOL1 G1 variant
ALANGIEEVHR	ALANGIEEVHR
ARLPVTTWR	ARLPVTTWR
LTATSIDR	LTATSIDR
TYAAIEDEYVQQK	TYAAIEDEYVQQK
	LTATSIDRLK
	VFKEVMR
	DITPNLLSL
	FTEEATKYFR
	IQESIEKLR

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/ndt.

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CONFLICT OF INTEREST STATEMENT

The results presented in this paper have not been published previously in whole or part, except in abstract format. D.J.F. and M.R.P. are named as co-inventors on patents related to *APOL1* diagnostics and therapeutics; they are also co-founders and own equity in APOLO1bio and receive research support from Vertex. Wake Forest University Health Sciences and B.I.F. have filed for a patent related to *APOL1* genetic

testing. B.I.F. and K.L.S. receive research support from Novartis Pharmaceuticals. B.I.F. is a consultant for Ionis Pharmaceuticals and AstraZeneca.

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