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Longitudinal Epigenome-Wide Analysis of Kidney Transplant Recipients Pretransplant and Posttransplant

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Introduction: Kidney transplantation remains the gold standard of treatment for end-stage renal disease (ESRD), with improved patient outcomes compared with dialysis. Epigenome-Wide Association Analysis (EWAS) of DNA methylation may identify markers that contribute to an individual's risk of adverse transplant outcomes, yet only a limited number of EWAS have been conducted in kidney transplant recipients. This EWAS aimed to interrogate the methylation profile of a kidney transplant recipient cohort with minimal posttransplant complications, exploring differences in samples pretransplant and posttransplant.

Methods: We compared differentially methylated cytosine-phosphate-guanine sites (dmCpGs) in samples derived from peripheral blood mononuclear cells of the same kidney transplant recipients, collected both pretransplant and posttransplant ($N = 154$), using the Infinium MethylationEPIC microarray (Illumina, San Diego, CA). Recipients received kidneys from deceased donors and had a mean of 17 years of follow-up.

Results: Five top-ranked dmCpGs were significantly different at false discovery rate (FDR) adjusted $P \leq 9 \times 10^{-8}$; cg23597162 within *JAZF1*, cg25187293 within *BTNL8*, cg17944885, located between *ZNF788P* and *ZNF625-ZNF20,* cg14655917 located between *ASB4* and *PDK4* and cg09839120 located between *GIMAP6* and *EIF2AP3*.

Conclusion: Five dmCpGs were identified at the generally accepted EWAS critical significance level of FDR adjusted P (P_{FDRadj}) $\leq 9 \times 10^{-8}$, including cg23597162 (within *JAZF1*) and cg17944885, which have prior associations with chronic kidney disease (CKD). Comparing individuals with no evidence of posttransplant complications ($N = 105$) demonstrated that 693,555 CpGs (89.57%) did not display any significant difference in methylation ($P_{FDRadj} \geq 0.05$), thereby this study establishes an important reference for future epigenetic studies that seek to identify markers of posttransplant complications.

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CKD is the 12th leading cause of mortality globally and is predicted to become the fifth leading cause of death by 2040.[1–3] CKD may progress to ESRD,[4] when renal replacement therapy options are considered, including kidney transplantation.[5] In Europe, 24,013 kidney transplants were performed in 2019,[6] compared with 24,273 kidney transplants in the United States (US, $n = 24{,}273$).[7] The United States Renal Data System 2020 annual data report highlighted the global challenge of ESRD with a prevalence of treated ESRD 2354 per million population in the US, 3587 per million population in Taiwan and 997 per million population in the UK (excluding Scotland).[8] Given the high cost to health care systems of performing kidney transplants, and the fact that the demand for kidney transplantation exceeds the combined supply of living and deceased donor kidneys, there is a need to improve kidney transplant outcomes. Posttransplant complications include infection (e.g., cytomegalovirus), allograft rejection (acute and chronic), cardiovascular disease, stroke, posttransplant diabetes mellitus, and malignancy (in particular, skin cancer). Kidney transplant recipients report that the lifelong care required for their allograft is a significant burden, which greatly impacts their quality of life.[9]

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Table 1. Participant characteristics

Characteristic	Pretransplant	Posttransplant
Number of participants	154	154
Number of females : males (%)	58 : 96 (38% : 62%)	58 : 96 (38% : 62%)
Mean age (± SD)	36.4 yr (14.4)	53 yr (13.5)
Primary renal diagnoses	Chronic renal failure ($n = 15$) Glomerulosclerosis ($n = 5$) Hereditary/familial nephropathy ($n = 5$) IgA nephropathy ($n = 19$) Polycystic kidney disease ($n = 25$) Pyelonephritis/interstitial nephritis ($n = 35$) Type 1 diabetes mellitus ($n = 9$) Others[a] with $n < 5$ individually ($n = 41$)	
CNI use in first 3 mo posttransplant	-	Ciclosporin: 104 Tacrolimus: 21[b]
Mean (± SD) follow-up time	-	17.0 yr (5.2)
Number of individuals who experienced allograft rejection	-	25 (16.2%)
Mean (± SD) graft survival time ($n = 129$) at time of posttransplant sample collection	-	17.1 yr (5.3)
Number of individuals who developed cancer at ≥1 site	-	49 (31.8%)
Number of individuals who developed skin cancer	-	16 (10.4%)

CNI, calcineurin inhibitors.
[a]Others include: Alport syndrome, amyloid, cortical tubular necrosis, cystinosis, congenital renal dysplasia, congenital renal hypoplasia, dense deposit disease, glomerulonephritis, Goodpasture's syndrome, Henoch-Schonlein purpura, lupus nephritis, membrano-proliferative glomerulonephritis type I, membrano-proliferative glomerulonephritis type II, rapidly progressive glomerulonephritis, renal vascular disease, tubulo interstitial nephritis, type 2 diabetes mellitus, and granulomatosis with angiitis.
[b]Remaining individuals who did not receive either ciclosporin or tacrolimus were given alternative forms of CNI which were not reported.

More than 50 candidate genes have been associated with adverse transplant outcomes in kidney recipients.[10–14] Yet, consideration of an individual's genetic profile is not sufficient to fully understand disease progression or predict adverse renal transplant outcomes. Epigenetic, or nonsequence level modifications such as DNA methylation, play a significant role in health and disease.[15] Epigenetic alterations provide a dynamic link between an individual's genetic background and the environment to which they have been exposed.[15,16] Differential methylation has important implications in several diseases that are leading causes of death globally, including CKD,[12] cardiovascular disease,[17] and type II diabetes mellitus.[18] Altered DNA methylation may influence the regulation of immune cells involved in allograft dysfunction, rejection, or risk of posttransplant complications.[19–21] Because DNA methylation is dynamic, it may be reversible. DNA methylation may provide potential targets for therapy, for example apabetalone has been explored as an epigenetic therapeutic for diabetic kidney disease (DKD).[22]

EWAS have been made possible by the development of high throughput microarrays, most recently enabling interrogation of approximately 850,000 sites across the epigenome.[23] High quality, longitudinal EWAS data remain scarce, particularly in the field of kidney disease research, with only 2 EWAS publications cited in the EWAS atlas.[24] EWAS have identified differential methylation associated with DKD in individuals with type 1 diabetes, CKD, and ESRD.[25–30] A limited number of EWAS have been published, which evaluate kidney transplant outcomes in recipients.[31–33] Transplantation results in significant molecular and metabolic alterations within recipients due several factors, such as the introduction of the foreign donor genome and the recipient's response to immunosuppressive medication.[34,35] Therefore, to identify clinically plausible epigenetic markers of adverse posttransplant outcomes, it is useful to first evaluate the baseline epigenetic profiles of individuals who have received reasonably successful transplants. The Northern Ireland Renal Transplant cohort has more than 50 years of harmonized, well curated longitudinal data of kidney transplant donors and recipients.[36]

This study aimed to compare DNA methylation profiles in the same individuals who received kidney transplants in Northern Ireland, before and after transplantation.

METHODS

Northern Ireland Renal Transplant Collection

All participants provided written informed consent. Existing DNA was used; DNA was frozen at −80 °C in multiple aliquots following extraction from whole blood using the salting out method and normalized using PicoGreen quantitation.[37,38] All participants ($N = 154$) were of European ancestry (Table 1). DNA samples were collected pretransplant for the purposes of tissue matching between 1986 and 2005, with excess diagnostic material stored and available for analysis. Posttransplant samples were collected

(between 2012 and 2014) with informed consent for longitudinal biomarker studies by convenience of those attending transplant clinics. The ethical approval reference numbers for the Belfast Renal Transplant samples are ORECNI 08/NIR03/79, 12/NI/0003, 12/NI/0178.

Laboratory Methodology

DNA was bisulphite treated using the EZ Zymo Methylation Kit (Zymo Research, Irvine, CA) following the Illumina Infinium HD Assay for Methylation Kit protocol.[39] All samples were prepared and analyzed using the Infinium MethylationEPIC Kit and BeadChips (Illumina, San Diego, CA) protocol.[40] Samples were processed through a consistent laboratory workstream with significant effort made to minimize unwanted variation through batch effects: same lot numbers used for consumables, minimization of freeze thaw cycles, and pretransplant and posttransplant samples randomly distributed across arrays.[41] Methylation arrays were scanned using a dedicated iScan machine (Illumina, San Diego, CA) with regular monitoring of laser intensity levels.

Quality Control (QC) and Data Analysis

Resulting .idat files were assessed using Illumina's BeadArray Controls Reporter software to assess QC and evaluate hybridization, extension, dye specificity, and bisulphite conversion.[40] Concordance of average β values for 7 duplicate samples was completed using GenomeStudio (Illumina, San Diego, CA) v1.8, methylation module including a sex check of all included individuals.

Proportional white cell counts (WCCs) were estimated using the Houseman method,[42] the minfi Bioconductor (v3.10) package and .idat files. Estimation of 6 peripheral WCCs was performed using the estimateCellCounts function with a *t* test used to compare WCC distributions between groups with a significance threshold of $P < 0.008$. QC, preprocessing, and differential methylation analyses were undertaken in the R statistical environment (3.6.3) utilizing RnBeads 2.0 and Bioconductor packages.[43] Cross-reactive probes and those located within 3 base pairs of common single nucleotide polymorphisms (SNPs) were excluded because of their ability to map to multiple areas of the genome and affect probe hybridization, respectively. Unreliable probes and samples were removed using the Greedycut algorithm ($P < 0.05$). Those located on sex chromosomes were also removed. Raw intensities were normalized using the bmiq method.

β values were generated and M values were derived. Association analysis directly compared methylation in the same individuals using samples collected both prerenal transplantation ($N = 154$) and postrenal transplantation ($N = 154$), adjusting for chronological age, sex, and proportional WCCs. In addition, methylation profiles were compared when excluding $n = 49$ individuals who developed posttransplant complications (with the same adjustments). Because rejection and posttransplant cancer were the 2 most common complications developed, subanalyses were conducted in these groups. Methylation profiles were evaluated in $n = 25$ individuals who developed rejection compared with $n = 129$ who did not and $n = 47$ individuals who developed cancer compared with $n = 106$ who did not. *P*-values were computed using the limma approach for each site with significance set at an FDR adjusted $P \leq \times 10^{-8}$.[44] Genome Studio v1.8, (Illumina, San Diego, CA) was used to ascertain the average number of beads per probe for the pretransplant and posttransplant sample groups and the average beta values for each significant dmCpG (FDR adjusted P [P_{FDRadj}] $\leq 9 \times 10^{-8}$). Hierarchical linear models from the limma package were employed and fitted using Bayesian approach on the derived M values.

All suggestively significant dmCpGs at $P_{\text{FDRadj}} \leq 9.9 \times 10^{-5}$, were reported with CpG locations mapped to Human Genome build 37. Significant dmCpGs ($P_{\text{FDRadj}} \leq 9 \times 10^{-8}$) were manually reviewed for SNPs, which may affect methylation based on the Infinium B5 manifest file (https://emea.support.illumina.com/downloads/infinium-methylationepic-v1-0-product-files.html), the Biobanking and Biomolecular Resources Research Infrastructure, the Netherlands database,[45] and dbSNP. CpG sites with SNPs having a minor allele frequency >1% located within 10 nucleotides of the target probe site were removed from downstream analyses.[46] Manhattan and quantile-quantile plots were drawn using the qqman package in R.[47]

Analyses of dmCpGs and Potential Overlaps With Transcription Factors

Previous investigations suggested that SNPs and transcription factors (TFs) influence methylation.[46,48] The top-ranked dmCpGs were examined using the eFORGE-TF database (https://eforge-tf.altiusinstitute.org/), to evaluate TF motif enrichment.[49] The dmCpGs were searched against previously acquired kidney data available in the online database ("fkidney" data set comprised 7 experiments). All significant motifs ($q \leq 0.01$) were reported.

Analysis of Gene Expression Profiles Using NephroSeq Transplant Datasets

Genes which contained significant dmCpGs located either side of the nongene-centric dmCpGs were searched

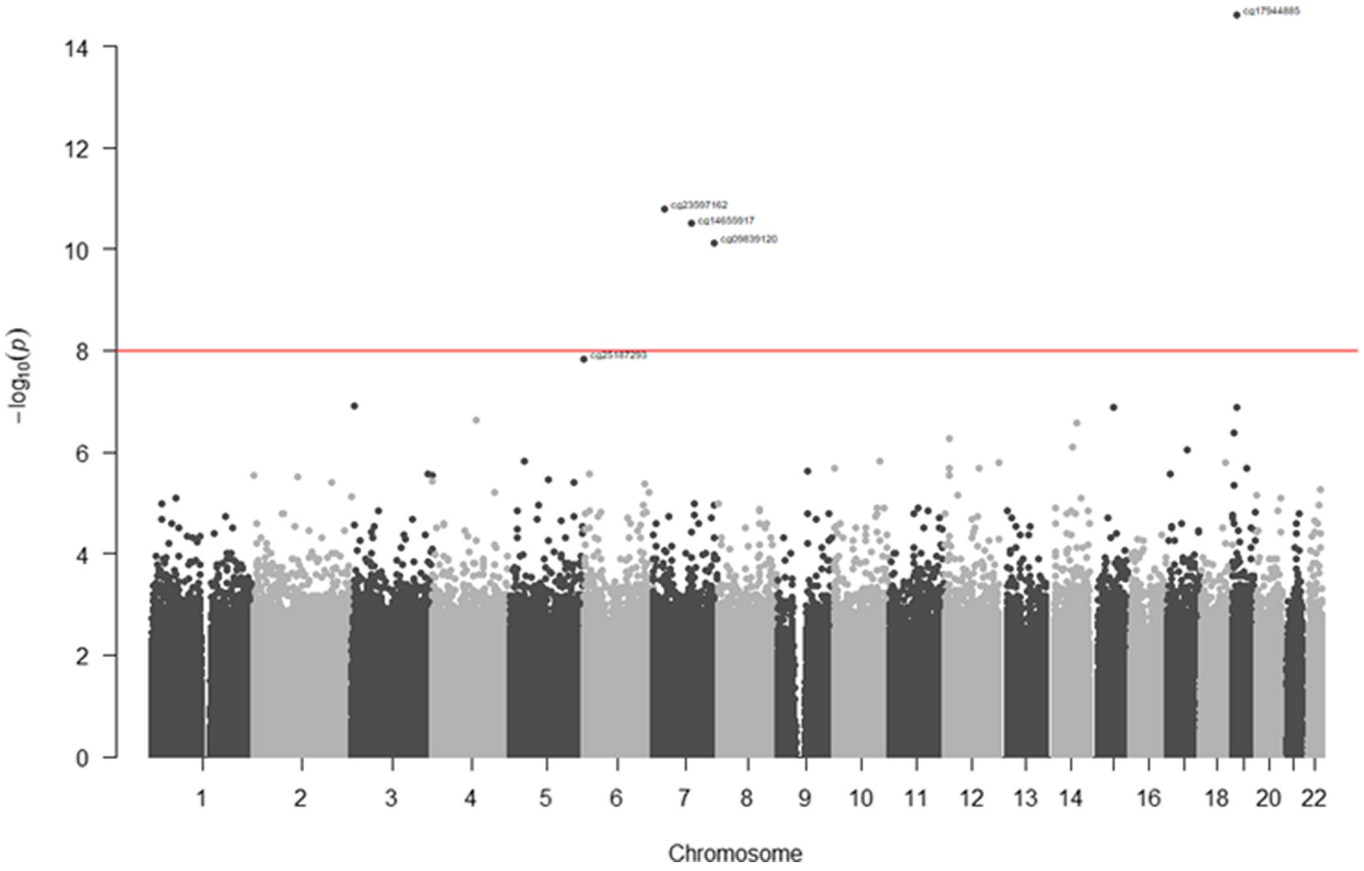

Figure 1. Manhattan plot of CpG sites drawn based on P_{FDRadj} values.

in NephroSeq v5 (https://www.nephroseq.org/).[50] Significant differential gene expression ($P \leq 0.01$; fold change (FC) $\pm$ 1.5) from "Disease vs. Control" analysis were reported.

Functional Analysis of Gene Ontology (GO), Pathways and Protein networks

Gene functionality was examined by GO enrichment and pathway analysis in genes containing dmCpGs with suggestive significance ($P_{FDRadj} \leq 9.9 \times 10^{-5}$). GO enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery v6.8, to identify enriched biological process, cellular component, and molecular function GO terms at $P < 0.01$, with a fold enrichment of at least ± 2, which included at least 3 genes (https://david.ncifcrf.gov).[51–54] Database for Annotation, Visualization, and Integrated Discovery was also utilized to identify any significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways, which included at least 3 genes with an enrichment threshold of at least ± 2 at $P \leq 0.01$.[55,56]

The Reactome Pathway Database (https://reactome.org/) was analyzed for enriched pathways at $P \leq 0.01$, which included at least 3 of the genes in which suggestive dmCpGs were located.[57] Computational predictions of protein interactions were generated using STRING v11 (https://string-db.org/).[58] Disconnected nodes were removed from the network, displaying only connected nodes with the highest confidence of interaction score (0.9).

RESULTS

Population Description

Of the 154 transplant recipients, 58 were females (38%) and 96 were males (62%). The average age of participants was 36 years pretransplant with a mean follow-up time of 17 years (range 9–28 years). Of the 154 participants, 49 (31.8%) developed posttransplant malignancy and 25 (16.2%) experienced allograft rejection. The number of participants included in each analyses is detailed in Supplementary Figure S1 as a Strengthening the Reporting of Observational Studies in Epidemiology diagram.

Data QC and Preprocessing

No samples failed analysis in BeadArray Controls Reporter (Supplementary Table 1). No significant difference was observed for proportional WCCs between pretransplant and posttransplant sample groups (Supplementary Table S2).

No samples were removed from this analysis during the QC or preprocessing steps performed via RnBeads.

Table 2. Statistically significant dmCpGs at $P_{FDRadj} \leq 9 \times 10^{-8}$

Probe ID	Gene	Genomic location	Probe site	P_{FDRadj}-value	UCSC gene region category	Average number of beads per probe	Nephroseq evidence (*P* value[a])
cg17944885	Between ZNF788P and ZNF625-ZNF20	19:12114860-12114981	19: 12225735	2.4×10^{-15}	Intronic	13.24 (All) 13.31 (pre-tx) 13.17 (post-tx)	0.007 (ZNF788P) 2.17×10^{-4} (ZNF625)
cg23597162	Within JAZF1	7:28062662-28062783	7: 28102341	1.63×10^{-11}	Gene body	9.72 (All) 9.96 (pre-tx) 9.47 (post-tx)	5.22×10^{-11} (JAZF1)
cg14655917	Between ASB4 and PDK4	7:95550298-95550419	7: 95179670	3.15×10^{-11}	Intronic	12.04 (All) 12.10 (pre-tx) 11.98 (post-tx)	5.25×10^{-9} (ASB4) 3.92×10^{-18} (PDK4)
cg09839120	Between GIMAP6 and EIF2AP3	7:150668851-150668972	7: 150365999	7.57×10^{-11}	Intronic	12.69(All) 12.71 (pre-tx) 12.66 (post-tx)	1.61×10^{-12} (GIMAP6)
cg25187293	Within BTNL8	5:180909216-180909337	5: 180336276	1.45×10^{-8}	TSS1500; Gene body	14.32 (All) 14.17 (pre-tx) 14.47(post-tx)	2.05×10^{-5} (BTNL8)

Adj, adjusted; FDR, false discovery rate; ID, identification; TSS, transcription start site; tx, transplant; UCSC, University of California Santa Cruz.
[a]Where more than one *P* value was ascertained for a specific site, the top-ranked *P* value is listed in this table.

Probes enriched for SNPs (17,371) were removed alongside 16,808 probes filtered by the Greedycut algorithm. Filtering, normalization of probes, and removal of those located on sex chromosomes (Supplementary Figures S2 and S3) resulted in 771,145 probes and all 308 samples included in association analysis. The quantile-quantile plot follows a normal distribution with a slight left skew (Supplementary Figure S4). Concordance of β values for 7 duplicate samples was completed, average $r^2 = 0.99$.

Identification of Top-Ranked dmCpGs

The epigenetic profiles were largely similar in pretransplant and posttransplant DNA samples from the same individual (Supplementary Figure S5, $r^2 = 0.999$). Comparison of the methylation patterns between blood samples collected for the same individuals both pretransplant and posttransplant identified 5 top-ranked dmCpGs ($P_{FDRadj} \leq 9 \times 10^{-8}$; Figure 1); cg23597162 within *JAZF1*; cg25187293 within *BTNL8*; cg17944885 between *ZNF788P* and *ZNF625-ZNF20;* cg14655917 between *ASB4* and *PDK4;* and cg09839120 between *GIMAP6* and *EIF2AP3* (Table 2, Supplementary Table S3).

The number of beads per probe for each of the 5 dmCpGs which met the EWAS threshold was >9.4 (Table 2). The average β values for pretransplant and posttransplant groups have been included for each of the 5 dmCpGs in Supplementary Figure S6. A further 278 dmCpGs showed suggestive significance ($P_{FDRadj} \leq 9.9 \times 10^{-5}$); 172 were located within known genes (Supplementary Table S3). Twelve dmCpGs ($P_{FDRadj} \leq 9.9 \times 10^{-5}$) were located within promoter regions. Analysis of TF motifs using the eFORGE-TF database identified that none of the top-ranked dmCpGs overlapped with TF binding sites.

Analysis of the 105 individuals who did not experience posttransplant complications (Table 2, $n = 49$ individuals developed posttransplant malignancy, and of these, $n = 25$ experienced allograft rejection), showed that 693,555 of 774,343 CpG sites (89.57%) displayed no significant difference in methylation ($P_{FDRadj} \geq 0.05$). The top 5 ranked differentially methylated CpG sites remained significant in the analysis excluding participants with posttransplant complications; (cg17944885: $P_{FDRadj} = 1.9 \times 10^{-10}$; cg14655917: $P_{FDRadj} = 1.6 \times 10^{-8}$; cg23597162: $P_{FDRadj} = 1.9 \times 10^{-8}$; cg25187293: $P_{FDRadj} = 1.2 \times 10^{-6}$; cg09839120: $P_{FDRadj} = 1.6 \times 10^{-6}$).

In the subanalyses of 25 individuals who experienced allograft rejection posttransplant compared with participants who did not, no dmCpGs were significant following FDR adjustment. One dmCpG site (cg04366076) was statistically significant at unadjusted ($P = 4.13 \times 10^{-7}$) and further 14 sites were suggestively significant ($P \leq 1 \times 10^{-5}$, Supplementary Table S4). These did not include any of the top 5 ranked dmCpGs from the primary analysis of the full participant cohort.

In individuals who developed posttransplant malignancy ($n = 47$), no dmCpGs were significant following FDR adjustment. One dmCpG site (cg24184919) was statistically significant at unadjusted ($P = 6.86 \times 10^{-6}$) and a further 5 sites were suggestively significant ($P \leq 1 \times 10^{-5}$, Supplementary Table S5). These did not include any of the top 5 ranked dmCpGs from the primary analysis of the full participant cohort.

Analysis of Gene Expression Profiles Using NephroSeq Transplant Datasets

Genes in which the 5 significant dmCpGs ($P_{FDRadj} \leq 9 \times 10^{-8}$) were located within or between, were further

investigated using NephroSeq; *ZNF788P* and *ZNF625-ZNF20* (cg17944885), *JAZF1* (cg23597162), *ASB4* and *PDK4* (cg14655917), *GIMAP6* and *EIF2AP3* (cg09839120), and *BTNL8* (cg25187293). Functional data were found for 7 of the 9 genes examined (Supplementary Table S6); *EIF2AP3* is a pseudogene and was therefore not covered by the NephroSeq analysis. No results were obtained for *ZNF20*.

ZNF788P showed an increase in FC (+6.2; $P = 0.007$) on comparing expression in renal biopsy samples collected from 5 individuals with CKD to 3 control biopsies collected from kidneys of healthy individuals.[59] RNA sequencing analysis for *ZNF625* using 31 renal biopsy tubulointerstitial samples from individuals with lupus nephritis compared with 5 healthy living donors revealed an FC of +1.7; $P = 2.47 \times 10^{-4}$.

Four alterations in gene expression were recorded for *JAZF1*, the most significant of which was $P = 5.22 \times 10^{-11}$, FC of +1.9 from renal biopsy samples comparing CKD ($n = 48$) to control biopsies ($n = 5$).[59] Two significant differences were seen for *ASB4* ($P = 5.25 \times 10^{-9}$; FC, +3.2 and $P = 0.006$; FC, +6.2), gained from the Nakagawa discovery and validation cohorts respectively.[59]

Twenty-four significant differences were observed for *PDK4*, the most significant of which compared 21 individuals with membranous glomerulonephritis to healthy individuals; $P = 3.92 \times 10^{-18}$; FC, −3.2.[60]

For *GIMAP6*, 13 significant changed gene expression were returned, the most significant of which was for CKD ($P = 1.61 \times 10^{-12}$; FC, +1.9).[59] The expression of *BTNL8* was significantly altered for CKD ($P = 2.71 \times 10^{-6}$; FC, −1.8) and DKD ($P = 2.05 \times 10^{-5}$; FC, −1.8) cohorts.[59,61]

In silico Functional Analysis

Eighteen enriched GO processes were identified, including 15 biological processes and 3 molecular functions.

Of the GO processes, several immunogenic activities were identified (Supplementary Table S7) including interleukin-12 production ($P = 0.0008$, fold enrichment +12.0), regulation of immune system process ($P = 0.0027$, fold enrichment +2.0) and immune response-regulating cell surface receptor signaling pathway ($P = 0.0045$, fold enrichment +3.1).

Encyclopedia of Genes and Genomes analysis did not return significant results. The Reactome database identified 2 pathways (Supplementary Table S8), estrogen-dependent nuclear events downstream of ESR-membrane signaling ($P = 4.77 \times 10^{-5}$), which involved 3 genes from our data set, *CCND1*, *BCL2*, *MAPK1*; and interleukin-4 and interleukin-13 signaling ($P = 0.0006$), which included 5 genes from our data set (*IL4R, CCND1, BCL2, RORA, CD36*).

Network analysis using STRING v11 connected 143 protein nodes linked by 31 edges (Supplementary Figure S7). No significant enrichment of protein interactions was identified.

DISCUSSION

Differential DNA methylation has been associated with CKD,[62] DKD,[28] ESRD,[27] IgA nephropathy,[63] lupus nephritis,[64] and polycystic kidney disease.[65] DNA methylation has also been considered in relation to kidney transplantation.[20,21] In this investigation, we identified a strong correlation ($r^2 = 0.999$) in the methylation profiles pretransplant and posttransplant within the same individuals who received largely successful kidney transplants. Analysis of individuals with no evidence of posttransplant complications demonstrated that 89.57% of CpG sites did not display any significant difference in methylation ($P_{\text{FDRadj}} \geq 0.05$). In the full analysis of 154 matched individuals pretransplant and posttransplant, 5 CpGs were identified as significantly different using stringent criteria ($P_{\text{FDRadj}} \leq 9 \times 10^{-8}$) between longitudinal samples compared pretransplant and posttransplant. The findings of this study are important for future epigenetic studies of postkidney transplant complications, because we have determined a baseline epigenetic profile that can be used for comparison.

Biological Significance of dmCpGs ($P_{\text{FDRadj}} \leq 9 \times 10^{-8}$)

The dmCpG identified with the strongest EWAS level of significance was cg17944885 ($P_{\text{FDRadj}} = 2.4 \times 10^{-15}$), located between *ZNF788P* and ZNF625-ZNF20 on chromosome 19. This CpG site has strong previous links to kidney disease, associates with estimated glomerular filtration rate in CKD from population-based studies ($P = 1.2 \times 10^{-23}$, $P = 8.7 \times 10^{-41}$),[26,66] DKD ($P_{\text{FDRadj}} = 2.0 \times 10^{-44}$)[67] and transethnic ($P = 1.24 \times 10^{-13}$) cohorts with sensitivity analyses confirming that this was not influenced by smoking, age, and body mass index.[30] Schlosser *et al.*,[66] also identified evidence for a functional role of cg17944885 through its association with the transcriptional regulator *ZNF439*. Differential methylation at cg17944885 was consistently associated with estimated glomerular filtration rate in blood and kidney biopsy samples. The underlying biological mechanism by which cg17944885 exerts an effect or is affected by declining renal function warrants further investigation.

Differential methylation has been reported in this study for cg23597162 (*JAZF1*) located on chromosome 7 ($P_{\text{FDRadj}} = 1.63 \times 10^{-11}$), with supporting gene

expression data from renal biopsy samples collected from 48 individuals with CKD compared with 5 with no evidence of renal disease ($P = 5.22 \times 10^{-11}$ and FC +1.9).[59] Differential methylation at cg23597162 was also associated with estimated glomerular filtration rate in CKD ($P = 2.8 \times 10^{-19}$) and human immunodeficiency virus ($P = 1.0 \times 10^{-4}$) studies.[26,68] Dayeh *et al.*,[69] assessed methylation of >470,000 CpG sites using pancreatic islets from 15 individuals with type II diabetes mellitus and 34 nondiabetic donor individuals, and identified 3 dmCpGs associated with type II diabetes mellitus within *JAZF1*, including cg23597162 ($P = 6.1 \times 10^{-5}$; $\Delta\beta - 2.79$). *JAZF1* affects gluconeogenesis, lipid metabolism, insulin sensitivity, and inflammation.[70] It is also thought to be a negative regulator of interferon gamma and interleukin-17 in macrophages, but has been solely examined in murine models.[70] Eleven individuals within our cohort had DKD, and this may be relevant to the development of posttrasplant diabetes mellitus. However, in 2009, Kang *et al.*,[71] assessed the association of type II diabetes mellitus risk gene variants and posttrasplant diabetes mellitus in 589 individuals, and found that the variant rs864745 within *JAZF1* was one of the major risk alleles, but that it was not significantly associated with posttrasplant diabetes mellitus. Therefore, the role of *JAZF1* and the development of posttrasplant diabetes mellitus remains unclear.

Cg14655917 is located between genes *ASB4* and *PDK4*. This dmCpG has not previously been linked to altered methylation patterns, or renal disease. Nevertheless, *ASB4* encodes a protein which is a member of the ankyrin repeat and SOCS box-containing family and has potential involvement in tumorigenesis.[72,73] CpG sites within this gene have been linked to pancreatic cancer, in which a decrease in the level of messenger RNA expression was also reported.[74] The identification of a tumorigenesis associated biomarker within our participant cohort was unsurprising given that 49 (31.8%) developed cancer within 17 years of receiving their transplant.

The mitochondrial protein coding gene *PDK4* is located on chromosome 7. The encoded protein is known to contribute to the regulation of glucose metabolism, and gene expression is regulated by insulin, glucocorticoids, and retinoic acid.[75,76] *PDK4* dysfunction may therefore plausibly be linked with insulin resistance and/or diabetes associated ESRD and renal transplantation. However, in this study we did not identify a significant difference in *PDK4* methylation between pretransplant and posttransplant individuals ($P = 0.16$). *GIMAP6* is located on chromosome 7 and encodes a member of the guanosine triphosphatases of immunity associated proteins, which may function in cell survival. Ho and Tsai,[77] reported that human *GIMAP6* is primarily expressed in T cells, with sensitivity to apoptosis and an acceleration in the activation of T cells. In a case study of 2 siblings with a predicted deleterious homozygous variant in *GIMAP6* and no expression of the GIMAP6 protein, both individuals reported accelerated apoptosis, but normal levels of lymphocytes.[78] Cg09839120 is located between *GIMAP6* and pseudogene *EIF2AP3*. Neither *GIMAP6* nor *EIF2AP3* have been linked to kidney disease or altered methylation patterns previously.[79]

Differentially methylated cg25187293 is located on chromosome 5, within *BTNL8* ($P_{\text{FDRadj}} = 1.45 \times 10^{-8}$) with changes in gene expression observed in NephroSeq where in the Nagawaka CKD Kidney discovery cohort ($P = 2.71 \times 10^{-6}$; FC, −1.8) and in an investigation by Woroniecka *et al.*,[61] comparing individuals with DKD to healthy living donors ($P = 2.05 \times 10^{-5}$; FC, −1.8).[59] *BTNL8* may be involved in the stimulation of primary immune response, including T-cell proliferation.[80–82] Altered methylation of this gene has not previously been reported in kidney disease, and therefore may reflect a novel epigenetic marker associated with immunologic alterations postkidney transplant.

In silico functional analyses revealed that several processes linked to immunology were significantly enriched, including interleukin-12 production, regulation of immune system process, immune response-regulating cell surface receptor signaling pathway, and regulation of immune response ($P < 0.01$; FC of at least ±2). This is unsurprising because of the use of immunosuppressants, including calcineurin inhibitors in over 80% of the individuals included in this study. In addition, both aging and cell aging processes were significantly enriched ($P < 0.01$; FC of at least ±2), which was expected because the mean follow-up time between the sample collections was 17 years.

Strengths and Limitations

Overall, this investigation has several strengths. This EWAS was conducted to ascertain the methylation profiles of 154 individuals who received a renal transplant, using DNA extracted from peripheral blood samples collected both pretransplant and posttransplant with systematic variation minimized. This is the largest EWAS study conducted for renal transplant recipients. Samples compared pretransplantation and posttransplantation are matched, being longitudinal samples obtained from the same individuals at 2 timepoints. We have utilized the most cost-effective, high-density methylation array available, the Infinium MethylationEPIC.[23] The methylation status was

assessed using blood-derived DNA samples from both timepoints, accounting for proportional WCCs, age, and sex of participants. We employed a very stringent significance threshold for dmCpGs ($P_{FDRadj} \leq 9 \times 10^{-8}$); to reduce the rate of false-positives in studies which use the Infinium MethylationEPIC array.[83] DmCpGs which gained a level of suggestive significance ($P_{FDRadj} \leq 9.9 \times 10^{-5}$) were included for the GO and pathway analyses to ascertain whether any of the genes in which the dmCpGs were located have had a cumulative effect.

This project sought to identify changes in methylation using a readily accessible biomarker source that could be sampled during routine clinic visits, hence the use of blood-derived DNA methylation. Several recent studies have shown that the PBMC methylome is effective in the identification of disease specific epigenetic biomarkers,[17,84,85] including in kidney disease.[26]

The inclusion of individuals with a wide range of primary kidney disease diagnoses reflects the nature of our Northern Ireland Transplant cohort. We sought a replication cohort with similar phenotypic criteria and long-term follow-up, but have been unsuccessful in identifying a similar cohort with longitudinal samples and/or methylation data.

A larger scale, multiomic analysis, which includes genetic variation, epigenetic alterations, and gene expression analyses on the same samples would be helpful to further determine the markers of interest for this phenotype and to improve understanding of the biological mechanisms involved.

CONCLUSION

Blood-derived DNA methylation levels in longitudinally collected samples were similar for most markers in these matched prekidney transplant and postkidney transplant recipients. Differentially methylated regions were identified within markers of CKD, including cg23597162 within *JAZF1* and cg17944885, with cg17944885 having particularly strong prior associations with estimated glomerular filtration rate and DKD.[30,66–68] We have established a reference epigenetic profile of a largely successful kidney transplant cohort with a mean allograft survival time of approximately 17 years.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

Study conception: AJM and LJS. Study design and manuscript drafting: AJM, LJS and KRK. Data aquisition, data analysis, and data interpretation: LJS and KRK. Data acquisition and data interpretation: JK and AM. Funding acquisition, data interpretation, manuscript review for intellectual content: APM and AJM. All authors and approved the submitted version of this manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary File (PDF)

Figure S1. Strengthening the Reporting of Observational Studies in Epidemiology flow diagram.

Figure S2. Histogram depicting the percentage of probes and samples removed and retained during the filtering process.

Figure S3. Highlighting the effect of the correction performed on the β values following normalization and filtering.

Figure S4. QQ plot drawn from the *P*-values generated from the methylation comparison of samples collected prerenal transplant and postrenal transplant.

Figure S5. Scatter plot showing the distribution of beta values per CpG site comparing pretransplant and posttransplant samples ($r^2 = 0.999$).

Figure S6. Distribution of average beta values for pretransplant and posttransplant populations for each of the 5 dmCpGs (FDR adjusted $P < 9 \times 10^{-8}$).

Figure S7. STRING v11 analysis of protein networks with the highest confidence interaction score.

Table S1. Illumina Bead Array Controls Reporter results for matched pretransplant and posttransplant samples.

Table S2. Proportional WCCs comparison between samples collected pretransplant and posttransplant.

Table S3. Top-ranked differentially methylated CpG sites derived from pretransplant versus posttransplant analysis for the same individuals ($N = 154$) FDR adj $P \leq \times 10^{-5}$.

Table S4. Top-ranked dmCpGs derived from subanalysis of rejection ($n = 25$) compared with no rejection ($n = 129$) posttransplant unadjusted $P \leq \times 10^{-5}$.

Table S5. Top-ranked dmCpGs derived from subanalysis of cancer development ($n = 47$) compared with no cancer ($n = 106$) posttransplant unadjusted $P \leq \times 10^{-5}$.

Table S6. NephroSeq analysis of genes in which top-ranked dmCpGs (FDR adj $P \leq \times 10^{-8}$) are located within or are close to.

Table S7. GO enrichment analysis for genes where differentially methylated CpG sites are located (FDR $P \leq \times 10^{-5}$).

Table S8. REACTOME pathway analysis for genes where differentially methylated CpG sites are located (FDR $P \leq \times 10^{-5}$).

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