

Understanding Diabetic Nephropathy – Is There a Genetic Susceptibility?

a report by

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Diabetes is rapidly increasing in prevalence,¹ resulting in profound socioeconomic impacts in both developed and developing countries. Furthermore, the parallel increase in the prevalence of complications of diabetes, particularly nephropathy, retinopathy and associated cardiovascular disease, is placing enormous demands on healthcare budgets.² Improved understanding of the aetiology and pathogenesis of these complications is urgently needed. Effective screening strategies to identify individuals with diabetes most likely to develop complications could improve outcomes by focusing resources on those at highest risk.

What Is Diabetic Nephropathy?

Diabetic nephropathy (DN) is a microvascular complication affecting patients with both type 1 and type 2 diabetes. It has become the leading cause of end-stage renal disease (ESRD) in Europe and the US managed by renal replacement therapy (kidney dialysis and/or renal transplantation).^{3,4} The clinical phenotype includes persistent proteinuria, a decreasing glomerular filtration rate (GFR) and hypertension.⁵ A sizeable minority of patients with diabetes eventually develop definite DN.⁶ The earliest clinical manifestation is microalbuminuria (incipient nephropathy), which may progress to overt proteinuria (dipstick urinalysis positive for proteinuria) followed by the emergence of hypertension, a declining GFR and, later, development of ESRD. Nevertheless, microalbuminuria is not an absolutely reliable predictor of progression to DN as some patients remain microalbuminuric while others show regression of albuminuria to normal albumin excretion rates.⁷ Patients with DN have much higher mortality rates than individuals with diabetes with normal albumin excretion rates. The excess mortality is largely attributed to higher rates of coronary artery disease, stroke and amputation,⁸ and the five-year survival rate for ESRD patients with diabetes is worse than for most cancers.

DN in type 1 and 2 diabetes differs in a number of ways. For example, compared with type 1 diabetes the rate of progression of renal failure in patients with type 2 diabetes is more variable. In patients with type 2 diabetes, microalbuminuria progression to advanced renal disease is less frequent and increases in blood pressure generally occur before onset of microalbuminuria.⁹ Older patients with type 2 diabetes may also have concurrent hypertensive renal vascular disease, and unlike patients with type 1 diabetes the age at diabetes onset is more difficult to establish. Therefore, phenotypic definition of DN is simpler in patients with type 1 diabetes, which is one reason why efforts to discover genetic variants associated with risk of DN might be more successful in these patients.

Pathophysiological features of DN include glomerular capillary hypertension, glomerular hyperfiltration, mesangial matrix expansion and glomerulosclerosis. Prolonged hyperglycaemia leads to chronic metabolic and haemodynamic changes that modify the activity of various intracellular signalling pathways and transcription factors.¹⁰ There is the subsequent induction of cytokines, chemokines and growth factors, particularly transforming growth factor beta (TGF β). These effects promote structural abnormalities in the kidney such as glomerular basement membrane thickening, podocyte injury and mesangial matrix expansion with the later development of irreversible glomerular sclerosis and tubulointerstitial fibrosis associated with declining GFR. The clinical management of DN includes optimal glycaemic control, treatment of dyslipidaemia and aggressive lowering of blood pressure ideally with angiotensin-converting enzyme (ACE) inhibitors and/or angiotensin II type 1 receptor (ARB) blockers.^{11–14}

Does Genetic Variation Contribute to Risk of Diabetic Nephropathy?

A number of factors can alter the risk of nephropathy in patients with diabetes (see *Table 1*). Intervention trials have demonstrated that excellent glycaemic control can reduce the risk of progression to nephropathy.^{11–13} However, hyperglycaemia alone is not responsible for development of DN, since some patients with diabetes do not develop nephropathy despite poor glycaemic control. Epidemiological studies have suggested that some individuals are 'protected' from DN because it generally develops within 15–20 years after diagnosis of diabetes or not at all.⁶ Family studies have demonstrated significant differences in recurrence risk for nephropathy in siblings with diabetes in which the proband had nephropathy compared with probands without nephropathy.¹⁵ The large differential in cumulative risk between these groups cannot be explained in terms of environmental factors alone. Certain ethnic groups (e.g. African-Americans, Hispanics and American Indians) are also at greater risk of DN, and there is clustering of hypertension, cardiovascular disease and dyslipidaemia among parents of patients with DN.^{16,17} Many of these characteristics also have a genetic basis. Segregation analysis in families with type 2 diabetes also suggests that genetic factors determine urinary albumin excretion levels.¹⁸ Collectively



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there is compelling evidence for a genetic predisposition to DN. Therefore, identifying susceptibility genes and causal variants is a major goal, as it should lead to prediction of those individuals with diabetes at low and high risk of nephropathy. It may also help identify new targets for therapy within molecular pathways involved in this serious complication of diabetes.

Candidate Gene-based Studies in Diabetic Nephropathy

A number of genetic association (case-control, and to a lesser extent family-based) and linkage studies have been conducted in DN, but replication of positive findings has proved inconsistent. Case-control association studies in DN generally assess the frequencies of single nucleotide polymorphisms (SNPs) in patients with diabetes with (cases) and without (controls) nephropathy for correlations with disease. Finding causal variants in these studies relies on the fact that there is non-random association of alleles (linkage disequilibrium) within the human genome. Thus, a positive association could mean that the finding is true and causal, or true because of linkage disequilibrium or indeed false by chance. Genotyping redundancy due to linkage disequilibrium has been exploited, notably in genome-wide association studies (GWAS), by the use of so-called tag SNPs (proxies) in order to have good genome coverage while minimising genotyping effort and cost.

Despite the lack of robust replication studies it is noteworthy that a large meta-analysis would tend to support involvement of the ACE insertion/deletion polymorphism in DN.¹⁹ The strategies employed and findings of many of these studies have been reviewed elsewhere.^{20–23} Many of the problems in identifying disease-causing genes in DN may be related to inappropriate phenotypic selection criteria and study design, especially statistically under-powered studies (both initial and replication) where there were small sample sizes or artefacts generated by population substructure. However, other factors have contributed, notably difficulties in obtaining genetic material from family members for linkage and family-based association studies because the disease is relatively late-onset. Furthermore, it has been suggested that there is likely to be heterogeneity for susceptibility genes in patients with proteinuria alone compared with those who progress to ESRD.²⁴

Genome-wide Association Studies

Recently, a wealth of GWAS have been published for a range of diseases (largely in populations of European ancestry)^{25–28} by the Wellcome Trust Case Control initiative. Studies employed approximately 2,000 cases and 3,000 controls, and identified known and unknown gene variant associations for several diseases, notably metabolic and cardiovascular disease,²⁶ autoimmune disease²⁷ and cancer.²⁸ The success of GWAS is due to improvements in the statistical power of studies, the availability of good SNP maps, advances in high-throughput genotyping platforms and computing power for bioinformatics and statistical genetics.²⁹

A recent analysis of published GWAS has (unsurprisingly) demonstrated that most common variants (minor allele frequency $\geq 5.0\%$) associated with disease have odds ratios of 1.2–1.5, whereas more penetrant rare variants have odds ratios ≥ 2.0 .³⁰ In many respects, GWAS have been successful, but for each disease the gene variants identified still explain only a proportion of the risk due to genetic susceptibility. Some of these limitations can be explained by the fact that GWAS are essentially based on the hypothesis that common disease is due to common variants,³¹ and that to detect common variants with small effects or rare variants (<5%) with large effects will require much larger sample sizes of cases and controls. However, there are ways to assist in finding new gene variants that include

Table 1: Key Risk Factors for Diabetic Nephropathy

Genetic predisposition/familial clustering
Poor glycaemic control
Hypertension
Microalbuminuria
Duration of diabetes
Ethnicity
Male sex
Smoking

meta-analyses of GWAS, and also analysis of data from GWAS in populations of non-European ancestry.^{32,33}

Genome-wide Association Studies in Diabetic Nephropathy

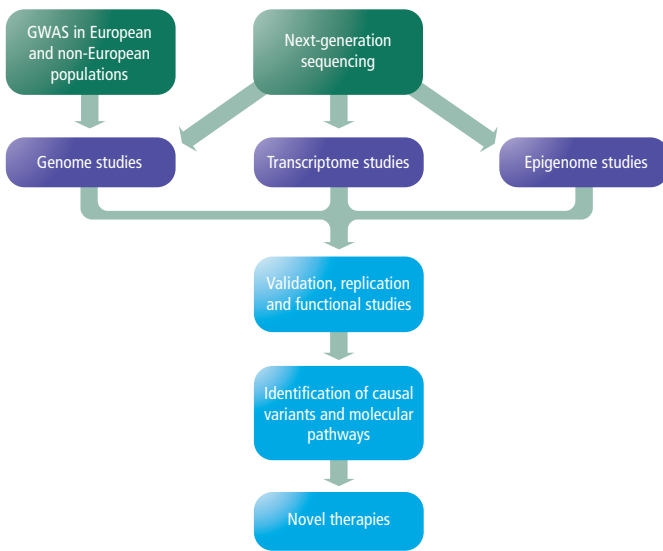
A GWAS for DN in type 1 diabetes has very recently been completed under the Genetic Association Information Network (GAIN) initiative³⁴ employing US Genetics of Kidneys in Diabetes (GoKinD)³⁵ study cases and controls and Affymetrix 5.0 500K SNP arrays. After exclusion criteria were applied, good-quality data were available for a total of almost 360,000 autosomal SNPs in 820 cases and 885 controls (AS Krolewski, personal communication). The two most promising SNP associations with DN to emerge from the primary analysis that approached genome-wide significance were rs10868025 (odds ratio [OR] 1.45; $p=5.0 \times 10^{-7}$), which is located near the 5' end of the FERM domain containing 3 (*FRMD3*) gene on chromosome 9q, and rs451041 (OR 1.36; $p=3.1 \times 10^{-6}$), located in an intronic region of the cysteinyl-tRNA synthetase (*CARS*) gene on chromosome 11p.

Imputation analysis (methods for deducing missing genotypes for untyped SNPs) identified an association with rs1888747 ($p=4.7 \times 10^{-7}$) on chromosome 9q, which was confirmed by genotyping this SNP in the US GoKinD case-control collection ($p=6.3 \times 10^{-7}$). Further analyses showed the 9q SNP associations to be associated with both proteinuria and ESRD, whereas rs451041 on chromosome 11p was associated with ESRD alone. Replication studies in Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications¹³ samples, which analysed time to nephropathy, supported the findings of the US GoKinD study; however, these replication data should be treated with caution as the study was statistically underpowered. In addition, expression analysis in human cell lines, including mesangial and renal proximal tubule cells, revealed high levels of expression for both *FRMD3* and *CARS* genes. Furthermore, immunohistochemistry analysis showed that *FRMD3* protein expression was increased in biopsy samples from patients with DN compared with controls. This GWAS represents a landmark in DN genetics, and is a starting point for the identification of causal gene variants in this complication (see *Figure 1*). Since the power of the US GoKinD GWAS is sufficient to identify common variants with large effects, the findings might therefore suggest that common variants with large effects are not involved in DN, but additional studies are required to confirm this. More GWAS in DN (both European and non-European ancestry) are urgently required to increase the statistical power to confirm initial findings and identify new variants. To assist in this effort, case control collections of similar size to that employed for the US GoKinD GWAS have been assembled including GoKinD UK/Warren3³⁶ and FinnDiane³⁷ collections.

Transcriptomics and Pathway-based Approaches

Expression profiling studies using *in vitro* and *in vivo* models of disease, and also human renal biopsies, have furthered understanding of the molecular drivers involved in DN. For example, recent studies have identified

Figure 1: Strategies for Identifying Therapeutic Targets



Genome-wide association studies (GWAS) and next-generation sequencing studies will improve knowledge of the molecular pathways involved in diabetic nephropathy, which should lead to the development of novel therapies.

approximately 200 genes to be differentially expressed in mesangial cells exposed to high levels of extracellular glucose.³⁸⁻⁴¹ Novel genes and gene transcripts identified include connective tissue growth factor (CTGF), and induced in high glucose-2 (IHG-2; Gremlin [GREM1]).

CTGF is a downstream mediator of TGFβ1-directed matrix production, and regulates actin cytoskeleton disassembly. Gremlin is a bone morphogenetic protein and is regarded as a novel target gene in DN.⁴² Recently, studies using bioinformatic analyses to identify genes with similar sequence and structure to *GREM1*, revealed significant similarity in both promoter and predicted microRNA (miRNA)-binding elements to the Notch ligand Jagged1 and its downstream effector, hairy enhancer of split-1 (Hes1).⁴³ Furthermore, TGFβ1 increased expression of these genes *in vitro*, and increased expression of Gremlin, Jagged1 and Hes1 was also found in renal biopsies from patients with DN. Upregulation of these genes was also found to co-localise to areas of tubulointerstitial fibrosis. These findings point to co-regulation of Gremlin and Notch signalling, and a possible new pathway implicated in DN. Genes identified by these approaches can provide interesting new candidates for genetic association studies in DN, particularly if they converge with findings from other studies such as GWAS. Also, expression profiling studies should assist in the identification of new therapeutic targets for intervention trials; this is particularly relevant since TGFβ itself is excluded as a suitable target due to its range of important actions.

Epigenetics and Diabetic Nephropathy

In addition to genetic variation, dysregulation of the epigenome can also lead to disease, notably in cancer,^{44,45} but also in other common diseases such as diabetes.⁴⁶ Epigenetic changes include DNA methylation (covalent attachment of methyl groups at CpG dinucleotides), histone modifications (acetylation, methylation, phosphorylation and ubiquitination) and RNA-based silencing.

DNA Methylation

DNA methylation influences gene regulation largely as a result of transcriptional silencing, and indirect evidence points to the involvement of DNA methylation in DN. For instance, alterations in DNA methylation may

be implicated in vascular disease,^{47,48} and characteristics known to be linked to DN such as hyperhomocysteinaemia, dyslipidaemia, inflammation and oxidative stress can promote aberrant DNA methylation.⁴⁹⁻⁵¹ The identification of aberrant DNA methylation in DN will provide new insights into the causes of this complication, and importantly could provide the basis for the development of novel treatments (e.g. DNA methylation inhibitors).

MicroRNAs

miRNAs are involved in post-transcriptional regulation of gene expression, and there is a growing awareness that altered (miRNA) expression is involved in disease.⁵²⁻⁵⁴ These non-coding RNAs (~21 nucleotides long) bind to the 3' untranslated regions (UTRs) of target genes and affect stability or translation of messenger RNA (mRNA), and may be implicated in DN.⁵⁵⁻⁵⁸ Of note, it has been shown that the 'C' allele of a SNP (1166A>C) in the 3' UTR of the *AGTR1* gene is functional in that it results in increased levels of AGTR1 by abrogating the regulation of hsa-miR-155.⁵⁶ Since the *AGTR1* 1166C allele has been reported to be associated with hypertension in many (but not all) studies, these findings could provide new targets for therapeutic treatments. Furthermore, a recent study has also demonstrated that miR-377 is elevated in experimental models of disease and that this indirectly leads to increased production of fibronectin, an important matrix protein in DN.⁵⁷

Therefore, comprehensive studies are required to assess altered expression of miRNA in both experimental models of disease and human biopsy material from affected and unaffected control individuals. Target genes for differentially expressed miRNAs can then be identified from established databases, and studies performed to assess if SNPs in the 3'UTR of target genes are associated with DN.⁵⁹ These new avenues of research may reveal more about the aetiology of DN.

Next-generation Sequencing and Future Approaches

The feasibility of cost-effectively and efficiently re-sequencing entire genomes of humans to identify causal gene variants has improved considerably with the recent development of next-generation sequencing technologies.^{60,61} Although the chemistries, throughput and cost per nucleotide base vary between these platforms, they all involve massive parallel sequencing and miniaturisation that provides ultra-high throughput (up to billions of bases per run) at a hugely reduced cost compared with standard dideoxy sequencing methods.^{62,63} Furthermore, with the ability to bar-code DNA samples (multiplexing)⁶⁴ and enrich for specific genomic region (e.g. exons) by array-based capture methods,⁶⁵ this could result in still further reductions in cost and improvements in throughput. Next-generation sequencing methods will be exceptionally useful in studying the genetic aetiology of DN (see Figure 1). For example, targeted re-sequencing of association signals within the genomic regions identified by GWAS will no longer be as cost prohibitive or inefficient to conduct. In addition, this technology has many other applications including transcriptome sequencing and the analysis of DNA methylation.

Concluding Remarks

It is abundantly clear that the identification of genetic and epigenetic risk factors for DN requires multidisciplinary approaches involving international collaborations. The need to increase sample sizes in case-control studies, to perform more GWAS informed by the results from expression profiling to assist SNP selection and conduct meta-analyses of available genotype data is now recognised. Additional case-control collections for replication of provisional positive associations from these scans will also be required to

confirm the reliability of the data. The challenge will be moving from association signals to causal variants and subsequent identification of new therapeutic targets in molecular pathways (see *Figure 1*). The application of ultra-high-throughput and cost-effective next-generation sequencing technologies will almost certainly revolutionise the ability of investigators to compare the genomes, transcriptomes and epigenomes of patients with diabetes with and without nephropathy. Armed with these new technologies and improved international collaboration to increase sample

sizes, there is a real sense that we have opportunities (hitherto unavailable) to ultimately identify the aetiology of DN and develop treatments for patients with this devastating condition. ■

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