European rational approach for the genetics of diabetic complications—EURAGEDIC: patient populations and strategy

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Abstract

Background. Diabetic nephropathy is likely to be a complex genetic trait. To date, most diabetic nephropathy candidate gene studies have tested a limited number of genes and variants in small sized populations, or in populations that were poorly matched or phenotyped. The main objective of the EURAGEDIC study was to address these problems.

Methods. Single nucleotide polymorphisms (SNPs) in candidate genes were tested for association with overt diabetic nephropathy (persistent albuminuria >300 mg/24 h) in a large (n = 2499) Type 1 diabetes case/control study. Testing for transmission disequilibrium in 541 independent parent–offspring trios with or without diabetic nephropathy was applied for validation of consistency. Candidate genes were selected based on previous linkage studies, knowledge of metabolic pathways, and animal models. A comprehensive SNP discovery in more than 100 candidate genes was performed by direct sequencing.

Results. In total, 1176 cases with diabetic nephropathy and 1323 diabetic controls with longstanding normoalbuminuria were included from three European populations (Denmark, Finland, France). Data were collected on HbA1c, blood pressure, urinary albumin excretion rate, kidney function, retinopathy, smoking, medication and cardiovascular disease. To summarize the relevant non-genetic predictors for diabetic nephropathy a baseline phenotypic model fitted to EURAGEDIC data included the covariates: sex, diabetes duration, HbA1c and smoking as well as pair-wise interactions.

Conclusions. The EURAGEDIC study is designed and powered to identify and validate common alleles as genetic risk factors for diabetic nephropathy in Type 1 diabetic patients.

Keywords: diabetic nephropathy; genetics; type 1 diabetes

Introduction

The increased mortality in patients with Type 1 diabetes is predominantly caused by the poor prognosis for patients with diabetic nephropathy (DN). DN is characterized by persistent albuminuria, raised arterial blood pressure, a relentless decline in glomerular filtration rate and is associated with high cardiovascular morbidity and mortality [1,2]. The pathogenesis of this devastating micro-vascular complication is, however, not fully understood, although a multifactorial aetiology has generally been considered [1]. Epidemiological and familial studies suggest that genetic factors influence the risk to develop DN. The familial clustering of DN [3,4], together with that of elevated blood pressure [5], diabetes [6], and increased cardiovascular morbidity and early mortality [7] supports the hypothesis, that hereditary factors are involved in the liability to develop DN.

Diabetic nephropathy is likely to be a complex genetic trait, that is, the disorder is the consequence of gene sequence variations at several genes in combination with unfavourable environmental factors.
The high cumulative incidence (30–40%) might be accounted for by the effect of combinations of predisposing alleles with relatively high frequency and modest effect at several independent loci. Despite rapid progress in sequencing the human genome and in molecular genetic and bioinformatic technologies during past decades, the success to map and identify genes responsible for complex traits has been modest so far.

To date, most DN candidate gene studies may have lacked the thoroughness and the sensitivity to detect association with complex traits, as they have tested a limited number of genes and variants in small sized populations. In addition, some study populations have been poorly matched or phenotyped, which frequently have resulted in lack of replication of the weak associations detected [2].

Therefore, the main objective of the European Rational Approach for the Genetics of Diabetic Complications (EURAGEDIC) study was to address these problems by a combination of comprehensive SNP (single nucleotide polymorphism) discovery in candidate genes; testing a number of SNPs in two large independent patient cohorts (first- and second-line); adjustment for relevant confounders; and analysis of haplotypes where possible. With this approach, we strive to further characterize the biological pathways involved in the pathogenesis of micro- and macrovascular complications of Type 1 diabetes, with a main focus on diabetic nephropathy.

The EURAGEDIC consortium comprises eight institutions from four different European countries combining all the necessary know-how and expertise to perform a comprehensive program of large-scale genetic studies of complex diseases. Within the consortium there are three clinical partners with access to large collections of well-characterized diabetic patients, an efficient technological platform for DNA analyses, expertise in statistical analysis for the studies in humans as well as expertise in genetic and physiological studies of rodent models.

Subjects and methods

Patient populations

Three European centres from Denmark [8], Finland [9] and France [10,11] contributed to the EURAGEDIC studies. All included subjects were of Caucasian descent.

Denmark. Since 1993 all Type 1 diabetic patients with diabetic nephropathy attending the Steno Diabetes Center in Copenhagen were asked to participate in a study of the genetics of diabetic complications. In total, 489 cases with established DN were enrolled. A control group of 463 longstanding Type 1 diabetic patients with persistent normoalbuminuria was recruited from the out-patient clinic. In addition, a nationwide survey of the Danish National Registry of Diagnosis was used to identify patients diagnosed with Type 1 diabetes and either diabetic kidney disease (DE102) or multiple complications (DE107).

After verification in patient records a letter of invitation was sent for all patients with a Type 1 diabetic sibling and/or two living parents available for investigation. A total of 217 families with a proband with DN or a normoalbuminuric Type 1 diabetic proband were investigated in the trio study.

Finland. The FINNDIANE study is an ongoing multicentre, nationwide collection of Type 1 diabetic patients and their relatives recruited from 56 participating diabetic referral centres. Patients are recruited irrespective of their disease duration or complications. All patients with an ascertained renal status were considered and from this collection 387 cases with established diabetic nephropathy and 469 normoalbuminuric controls with duration of diabetes of >15 years were available for the case-control study. In addition, 202 pedigrees were classifiable for inclusion in the trio study. All probands fulfilled the EURAGEDIC definitions of cases and controls.

France. Caucasian Type 1 diabetic patients participated in a cross-sectional study for the genetic study of diabetic complications, conducted in 17 diabetes clinics in France and Belgium between 1994 and 1995. In addition, a nationwide collection of Type 1 diabetic patients and their relatives was performed. In total, 300 cases and 391 controls were recruited. Furthermore, 122 families were available for the trio study. All probands fulfilled the EURAGEDIC definitions of cases and controls.

Non-diabetic control subjects

Three non-diabetic cohorts from the general populations (n = 736) were used to explore national differences in allele frequency (see details in Supplementary Materials).

The study was approved by the ethical committees in each of the participating countries, and all subjects gave written informed consent.

Definitions

Type 1 diabetes was considered present if the age at onset of diabetes was ≤35 years and the time to definitive insulin therapy ≤1 year.

Established diabetic nephropathy (cases) was defined by persistent albuminuria (>300 mg/24 h or >200 μg/min or >200 mg/l) in two out of three consecutive measurements on sterile urines, after >5 years diabetes duration. In patients with ongoing angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARB) the last measurement of urinary albumin excretion before treatment initiation was used for classification. Patients with clinical suspicion of non-diabetic renal or urinary tract disease were excluded.

Absence of diabetic nephropathy (controls) was defined as persistent normoalbuminuria (urinary albumin excretion rate: <30 mg/24 h or <20 μg/min or <20 mg/l) after at least 15 years of diabetes duration in patients not treated with ACE inhibitors or ARBs.

Clinical characteristics

A minimal set of clinical parameters available at the three sites at the time of inclusion (cross-sectional data) was defined including age, age at diabetes onset, height, weight, insulin dose, HbA1c, systolic blood pressure, diastolic blood...
pressure, antihypertensive treatment, urinary albumin excretion rate, serum/plasma creatinine, renal replacement therapy, retinopathy, smoking status and cardiovascular complications (stroke, myocardial infarction, amputation).

In the majority of patients urinary albumin excretion rate was measured on timed urine samples in a central laboratory by validated methods, i.e. enzyme immunoassay (Denmark), radioimmunoassay (Finland) and nephelometry (France). The remaining 16% of the French patients \(n=88\) were classified based on repeated measurements of urinary concentrations of albumin in a morning spot urine sample.

Blood pressure was measured twice in the resting state with a standard sphygmomanometer and appropriate cuff size. HbA1c was analysed by standard high performance liquid chromatography (HPLC) techniques locally with normal values in the range from 4% to 7% in all centres. Serum/plasma creatinine concentration was determined by a modified Jaffe’s method centrally in each country. For descriptive purposes, the last available creatinine data was considered, even in patients who had previously had a kidney graft. Renal replacement therapy was defined as kidney transplantation or dialysis. Patients in renal replacement therapy were classified as cases, irrespective of their actual creatinine concentration. Retinopathy was graded based on fundus photography or direct ophthalmoscopy by an experienced ophthalmologist as nil, simplex or proliferative fundus photography or direct ophthalmoscopy by an experienced ophthalmologist as nil, simplex or proliferative retinopathy. Former and current smokers of one or more cigarettes per day were classified as smokers, all others as non-smokers. Cardiovascular disease was considered present in patients with a history of admission for stroke, myocardial infarction or vascular amputations.

Study design

The EURAGEDIC study design involved two phases for the genotyping of DNA variants, a first-line case-control study and a second-line trio study. The analysis strategy is a two-stage sequential design with stopping for futility. All selected variants were tested in the first-line study, and further in the second-line cohorts if the results of the first-line univariate analyses yielded a \(P\)-value \(\leq 0.1\). The power of the first-line study was 71% for a genotype risk ratio (GRR) = 1.3 and a minor allele frequency of \(\geq 0.25\) with an \(\alpha\) of 0.000125. To maximize the power of the first-line case-control study, 119 cases and 196 controls from the trio studies were included. When markers were genotyped in both the first and second-line studies these probands were solely included in the trio analyses.

For the trio study, families with at least one Type 1 diabetic proband with DN or with persistent long-lasting normoalbuminuria were considered and trios with both parents were recruited. When only one parent was available, the probands’ siblings were recruited in order to be able to infer probabilities for the missing parental genotype. The inclusion of trios with Type 1 diabetic probands affected or unaffected with DN allows us to search for opposite transmission patterns and reduces the risk of Type 1 diabetes loci from being detected [12].

Selection of candidate genes

The study is a systematic investigation of more than one hundred candidate genes identified through studies of susceptibility loci suggested by linkage studies, knowledge of metabolic pathways and data from animal models [13] (Supplementary Material—Table).

Positional candidate genes. Identified through several whole genome screens performed for Type 1 and 2 diabetes, insulin resistance, obesity, vascular complications of diabetes, blood pressure and hyperlipidaemia. Regions of particular interest include regions on chromosome 3q, 7q, 18q and 20p [14–19].

Functional candidate genes. Selected based on existing knowledge of metabolic pathways involved in the pathophysiology of diabetic complications. Among several options, three major avenues of investigation have been identified: (i) genes involved in glucose metabolism, including diabetes susceptibility genes or metabolism of glucose in target tissues, (ii) genes involved in the risk for vascular disease in the general population, including genes regulating blood pressure and cardiovascular risk and (iii) genes involved in the structure and growth of target tissues.

Animal models. New candidate genes are identified through the characterization of genes that exhibit altered transcriptional levels in the kidneys of diabetic and control rats ( GK-rat) and mice ( ENU mouse). Furthermore, the validation of the effect of impaired glucose homeostasis on the expression of these genes is ongoing. Thereby, new genes and pathways involved in the pathogenesis of diabetic complications in animal models are revealed. With the progress of the comparative genetic maps of rat, mouse and human genomes the human homologues of genes identified in the pathogenesis of diabetic complications in rodents will become available.

Single nucleotide polymorphism (SNP) discovery and SNP selection for genotyping

Genes selected for study were examined for polymorphisms from databases and by a SNP discovery platform. All exons and flanking intron sequences, 5’ and 3’ untranslated regions as well as promoter regions of each gene were screened by direct sequencing, using two sets of pooled DNA (see Supplementary Material for details). Sequencing reactions were performed according to the dye terminator methods using an ABI PRISM 3700 Analyzer (Applied Biosystems, Foster City, CA, USA). Alignment of experimental results, SNP discovery and genotyping were performed by the Genalys Software [20]. Haplotype structure and frequencies were determined from data concerning pooled DNA using the expectation maximization algorithm [21]. SNPs tagging the most frequent haplotypes (at least 5% in one population) were selected for genotyping. In addition, all non-synonymous variants that were detected in at least one diseased population were systematically investigated. We also examined 94 SNP genomic control markers in non-genic regions spaced throughout the genome to control for possible stratification within each population.

SNP genotyping was performed at the CNG (Centre National de Genotypage, Evry, France) using automated high throughput methods. Single SNP genotyping methods include the TaqMan, Amplifluor and MALDI-mass spectrometry platforms, whereas SNPllex method was used for multiplex SNP genotyping. All liquid handling was performed robotically in 384 well plates.
A success rate of >85% for genotyping was accepted. This threshold was selected to balance the loss of genotypic information with the relatively large efforts needed to retype individual samples using high-throughput technologies. Furthermore, quality control included 192 replicate samples genotyped blindly, DNAs from both cases and controls on each plate and to check for Hardy–Weinberg equilibrium in cases and controls, in each population, and on each individual plate. For trios, microsatellite markers (Panel 16, LMSV2, Applied Biosystems, Courtaboeuf, France) were genotyped to verify the family relationship. Nine trios that exhibited genotype patterns which were incompatible with the putative family structure were excluded.

Due to low DNA resource left, DNA sharing is not possible through the EURAGEDIC project, but data sharing is welcome to improve scientific knowledge. Requests should be addressed to the corresponding author to consider collaborations.

Statistical analyses

All phenotypic data are presented as mean (SD), except for serum creatinine and urinary albumin excretion rate, which due to their non-normal distribution are expressed as geometric means with 95% confidence intervals (CI) and log transformed before analysis. Comparisons of continuous baseline phenotypic covariates between cases and controls were performed with unpaired Student’s t-test or a non-parametric test of equality of medians. Chi-squared test statistic was employed to compare categorical variables between cases and controls. Test of homogeneity of covariates across the three populations was performed using one-way ANOVA (Analysis of variance) or Chi-squared test as appropriate.

To summarize the relevant non-genetic predictors for diabetic nephropathy in the EURAGEDIC cohorts multivariate logistic regression models were fitted to data to obtain the baseline phenotypic model (BPM). The covariates used are sex, diabetes duration (in years), HbA1c and smoking status. The ‘survey’ suite of STATA procedures was used for this analysis using population (France, Denmark and Finland) to define the model strata; thus the BPM presents a (linear) population-weighted model of disease risk relevant for the EURAGEDIC samples. Model selection was carried out using a hierarchical backward elimination procedure with main and two-way interaction terms. A further series of models was fitted to each population group separately to allow the risks associated with differing levels of covariates to vary across the three populations.

After obtaining the BPM the effect of each genetic marker will be considered in the light of the BPM. That is, for each marker a logistic regression model will be fitted to estimate its odds ratio (OR) adjusting for the covariates in the fitted BPM.

A post-hoc power calculation for the first- and second-line studies combined (Table 1), using a multiplicative model of genetic risk and an \( \alpha = 0.000125 \), revealed that the EURAGEDIC study has good power (>85%) to detect relatively small genetic differences: genotype risk ratio, GRR = 1.3 for an allele frequency > 0.25. Multiple testing error will be corrected for using the effective number of independent tests after taking into account linkage disequilibrium between markers [13].

The STATA (versions 7 and 9) statistical package was used to perform the analyses.

Results

First line case-control study

Clinical characteristics of Type 1 diabetic probands included in the case-control and the trio study are presented in Tables 2 and 3, respectively. In Table 2, the clinical characteristics of the subjects included in the first-line study are shown for each country separately.

Baseline phenotypic model

The baseline phenotypic model (BPM) predicts risk of DN within the EURAGEDIC cohorts; these risks are a weighted-average across the three populations (Table 4). This model shows that male sex, long duration of diabetes, high HbA1c level and smoking are significant \( (P < 0.007) \) main-effect predictors of developing DN in the EURAGEDIC cohort. There is also some evidence \( (P = 0.007) \) of an interaction between HbA1c level and diabetes duration. Analysis of each population shows that there are a few important differences in covariate-predicted risks across the three populations (Table 5), most notably with HbA1c levels. Mindful of the principle of parsimony and stressing the desire to model European-wide risk for DN, we propose that the EURAGEDIC BPM for the combined data set is appropriate for modeling individual-specific risks in these populations.

Second line trio study

For the second-line study, 253 trios of probands with established nephropathy and 288 control trios were studied—clinical data are presented in Table 2.

As in the first-line case-control study, trio probands with nephropathy had poorer glycaemic control, higher blood pressures and elevated creatinine, received antihypertensive medication and smoked more frequently and had more severe retinopathy
### Table 2. Clinical characteristics of 2499 Type 1 diabetic patients with and without diabetic nephropathy included in the first-line case-control study

|                      | Denmark (n = 952) | Finland (n = 856) | France (n = 691) | Combined (n = 2499)* | P-value
cases | controls | cases | controls | cases | controls | cases | controls | cases | controls | cases | controls |
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<tr>
<td>Sex (male/female)</td>
<td>293/196</td>
<td>247/216</td>
<td>227/160</td>
<td>165/135</td>
<td>685/491</td>
<td>631/692</td>
<td>&lt;0.0001</td>
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<td>Age (years)</td>
<td>41.8±10.3</td>
<td>45.1±11.3</td>
<td>41.3±9.3</td>
<td>43.1±11.2</td>
<td>42.0±10.2</td>
<td>44.8±11.0</td>
<td>&lt;0.0001</td>
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<td>Age at onset of diabetes (years)</td>
<td>13.9±8.2</td>
<td>17.8±8.7</td>
<td>12.1±6.9</td>
<td>14.3±7.8</td>
<td>15.6±8.9</td>
<td>13.6±7.9</td>
<td>15.9±8.6</td>
<td>&lt;0.0001</td>
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<td>Diabetes duration (years)</td>
<td>27.9±8.7</td>
<td>27.3±10.1</td>
<td>29.2±8.1</td>
<td>29.8±7.9</td>
<td>28.8±9.2</td>
<td>30.0±8.7</td>
<td>28.4±8.7</td>
<td>29.2±9.0</td>
<td>0.20</td>
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<td>BMI (kg/m²)</td>
<td>24.1±3.4</td>
<td>24.2±3.5</td>
<td>25.3±3.9</td>
<td>24.7±3.8</td>
<td>24.4±3.6</td>
<td>24.6±3.3</td>
<td>0.15</td>
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<td>Insulin Dose (IU)</td>
<td>44±15</td>
<td>42±14</td>
<td>52±18</td>
<td>41±13</td>
<td>46±16</td>
<td>44±14</td>
<td>&lt;0.0001</td>
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<td>HbA1c (%)</td>
<td>9.4±1.5</td>
<td>8.4±1.1</td>
<td>8.9±1.6</td>
<td>8.6±1.8</td>
<td>9.0±1.6</td>
<td>8.3±1.2</td>
<td>0.0007</td>
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<td>Systolic BP (mmHg)</td>
<td>145±22</td>
<td>134±19</td>
<td>147±22</td>
<td>144±19</td>
<td>145±21</td>
<td>132±17</td>
<td>&lt;0.0001</td>
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<td>Diastolic BP (mmHg)</td>
<td>83±12</td>
<td>76±9</td>
<td>84±11</td>
<td>82±11</td>
<td>83±11</td>
<td>76±9</td>
<td>&lt;0.0001</td>
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<td>Antihypertensive medication</td>
<td>67%</td>
<td>14%</td>
<td>92%</td>
<td>82%</td>
<td>79%</td>
<td>22%</td>
<td>&lt;0.0001</td>
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<td>UAER (mg/24h)f</td>
<td>526 (458–603)</td>
<td>111 (106–116)</td>
<td>356 (293–432)</td>
<td>268 (202–356)</td>
<td>393 (348–443)</td>
<td>8 (8–9)</td>
<td>&lt;0.0001</td>
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<td>Serum creatinine (moll/l)</td>
<td>3.3</td>
<td>24.1</td>
<td>3.1</td>
<td>24.3</td>
<td>0.0001</td>
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<td>Renal replacement therapy</td>
<td>7/139/343</td>
<td>166/173/124</td>
<td>14/36/330</td>
<td>0/17/283</td>
<td>21/192/956</td>
<td>309/478/531</td>
<td>&lt;0.0001</td>
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<td>Retinopathy (nil/simplex/proliferative)</td>
<td>66%</td>
<td>64%</td>
<td>56%</td>
<td>38%</td>
<td>0.0001</td>
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<td>Smokers</td>
<td>10%</td>
<td>4%</td>
<td>23%</td>
<td>16%</td>
<td>16%</td>
<td>5%</td>
<td>&lt;0.0001</td>
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<tr>
<td>Cardiovascular disease</td>
<td>67%</td>
<td>14%</td>
<td>92%</td>
<td>82%</td>
<td>79%</td>
<td>22%</td>
<td>&lt;0.0001</td>
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Data are n, mean ± SD, or geometric mean (95%CI).  
*With the exception of sex, duration of diabetes and blood pressure in cases, all covariates show significant heterogeneity across populations, P < 0.01.  
†P-value for test of difference between cases and controls.

### Table 3. Clinical characteristics of 541 type 1 diabetic probands with and without diabetic nephropathy included in the second-line trio study

|                      | Denmark (n = 217) | Finland (n = 202) | France (n = 122) | Combined (n = 541)* | P-value
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<tr>
<td>Sex (male/female)</td>
<td>72/64</td>
<td>43/38</td>
<td>30/25</td>
<td>21/26</td>
<td>140/113</td>
<td>141/147</td>
<td>0.14</td>
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<tr>
<td>Age (years)</td>
<td>36.6±7.3</td>
<td>38.6±8.8</td>
<td>39.6±8.2</td>
<td>40.4±9.2</td>
<td>38.4±6.0</td>
<td>35.2±9.3</td>
<td>0.12</td>
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<tr>
<td>Age at onset of diabetes (years)</td>
<td>10.3±6.1</td>
<td>12.6±6.9</td>
<td>13.5±8.1</td>
<td>12.1±7.2</td>
<td>11.7±5.8</td>
<td>10.6±6.1</td>
<td>11.3±6.7</td>
<td>11.9±6.9</td>
<td>0.35</td>
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<tr>
<td>Diabetes duration (years)</td>
<td>26.5±7.5</td>
<td>26.0±7.4</td>
<td>26.1±7.7</td>
<td>27.4±7.0</td>
<td>26.6±5.8</td>
<td>24.7±7.5</td>
<td>26.5±7.2</td>
<td>26.3±7.3</td>
<td>0.84</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.1±3.3</td>
<td>24.1±2.7</td>
<td>24.2±3.7</td>
<td>24.4±3.4</td>
<td>25.0±3.8</td>
<td>24.5±3.1</td>
<td>0.98</td>
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<tr>
<td>Insulin Dose (IU)</td>
<td>47±14</td>
<td>44±13</td>
<td>45±19</td>
<td>46±15</td>
<td>55±25</td>
<td>49±16</td>
<td>0.14</td>
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<tr>
<td>HbA1c (%)</td>
<td>9.1±1.5</td>
<td>8.5±1.1</td>
<td>8.2±1.5</td>
<td>8.3±1.2</td>
<td>8.7±1.8</td>
<td>7.9±1.1</td>
<td>8.8±1.6</td>
<td>8.3±1.2</td>
<td>&lt;0.0001</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>141±20</td>
<td>129±13</td>
<td>148±24</td>
<td>126±16</td>
<td>144±23</td>
<td>125±14</td>
<td>&lt;0.0001</td>
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<tr>
<td>Diastolic BP (mmHg)</td>
<td>83±11</td>
<td>75±9</td>
<td>84±11</td>
<td>74±8</td>
<td>83±11</td>
<td>79±7</td>
<td>&lt;0.0001</td>
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<tr>
<td>Antihypertensive medication</td>
<td>76%</td>
<td>14%</td>
<td>91%</td>
<td>19%</td>
<td>97%</td>
<td>10%</td>
<td>&lt;0.0001</td>
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<tr>
<td>UAER (mg/24h)f</td>
<td>428 (318–576)</td>
<td>104 (97–110)</td>
<td>117 (57–236)</td>
<td>10 (9–12)</td>
<td>455 (226–916)</td>
<td>11 (8–14)</td>
<td>0.0001</td>
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<tr>
<td>Serum creatinine (moll/l)</td>
<td>0.8%</td>
<td>3%</td>
<td>17%</td>
<td>5%</td>
<td>15%</td>
<td>0%</td>
<td>15%</td>
<td>1%</td>
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<tr>
<td>Retinopathy (nil/simplex/proliferative)</td>
<td>65%</td>
<td>54%</td>
<td>50%</td>
<td>38%</td>
<td>61%</td>
<td>33%</td>
<td>59%</td>
<td>42%</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>8%</td>
<td>3%</td>
<td>17%</td>
<td>2%</td>
<td>15%</td>
<td>0%</td>
<td>15%</td>
<td>1%</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
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</tbody>
</table>

Data are n, mean ± SD, or geometric mean (95%CI).  
*There is significant heterogeneity across populations with respect to age, haemoglobin A1c and retinopathy and for age at onset, insulin dose, creatinine, renal replacement therapy and antihypertensive medication in cases and for diastolic blood pressure and smoking in controls.  
†P-value for test of difference between cases and controls.  
‡UAER (urinary albumin excretion rate)—some patients with previous persistent macroalbuminuria had UAER below 300 mg/24 h at investigation due to ongoing antihypertensive medication.
and cardiovascular disease as compared with normoalbuminuric Type 1 diabetic controls.

Applying a trio design implies that parents are alive and available for investigation. Therefore, as expected, patients in the second-line study were on average 6 years younger, had developed diabetes earlier and had shorter duration of diabetes in comparison with probands of the first-line study, $P < 0.0001$.

Discussion

The EURAGEDIC study is a large case-control study including more than 1000 Type 1 diabetic case-control pairs from three European countries (Denmark, Finland, France). Testing for transmission disequilibrium in 541 independent parent-offspring trios with or without diabetic nephropathy from the three countries is used to evaluate positive results from the case-control cohorts using a design and statistical analysis that are robust to population stratification and provide an opportunity to evaluate parent-of-origin effects. Our aim is to identify genetic susceptibility to DN through a strategy based upon comprehensive SNP discovery in a large number of candidate genes, testing a large number of SNPs, adjustment for relevant confounders, and analysis of haplotypes.

Besides good glycaemic control, the attenuation of the renin-angiotensin system with ACE inhibitors or ARB is important in the clinical management of early diabetic kidney disease. It was, therefore, not surprising that the study of candidate genes for DN began in 1994 with the study by Marre et al. [22] reporting a protective effect of the II genotype of the ACE-gene for the development of this complication. Since then a considerable number of reports on the association of the ACE/ID polymorphism and DN have been published and now, a decade after the initial paper, more than 50 studies have been carried out in different patient populations with predictable conflicting results [23].

Contributing to the confusion are major differences between studies in criteria for the diagnosis of diabetic kidney disease. The majority of previous studies includes patients with both micro- and macro-albuminuria among cases, despite existing studies [24] demonstrating that Type 1 diabetic patients with micro-albuminuria may not necessarily progress to overt DN. To reduce case heterogeneity in the EURAGEDIC study, patients with micro-albuminuria were not included and strict criteria for established DN have been applied in both the first-line and second-line study.

Selection of control subjects for the present study was similarly strict and required patients to have had Type 1 diabetes and persistent normoalbuminuria for more than 15 years, thereby minimizing the number of controls who will in the future eventually develop severe diabetic kidney disease. Furthermore, blockers of the renin-angiotensin system with the potential of urinary albumin excretion lowering were not allowed for in the control groups to avoid misclassification.

Although strict pre-defined phenotypic inclusion criteria were agreed upon, several interesting differences in clinical characteristics exist between the three patient populations in the EURAGEDIC study. Since different strategies for patient recruitment have been applied in the three countries [8–11], sampling bias is likely to contribute to differences in nephropathy risk, retinopathy and renal replacement therapy patterns. To adjust for possible heterogeneity of gene-associations across populations, it was decided to perform tests for allelic association with disease for each of the three populations separately and to combine results in a weighted analysis. Furthermore, for each polymorphism a logistic regression model was fitted to estimate its odds ratio adjusting for the covariates and their interactions in the fitted BPM. The BPM was selected to be predictive of DN for the EURAGEDIC cohorts, and is thus only of relevance to the interpretation of this study. Interestingly, the analysis of clinical characteristics clearly identified a consistent association between smoking and DN, both in the first- and second-line cohorts, which was not fully established hitherto in type 1 diabetic patients [25,26].

Table 4. Summary of the fitted baseline phenotypic model (BPM) selected to be predictive for the EURAGEDIC cohorts – each genetic marker will be considered in the light of this EURAGEDIC BPM

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>1.57</td>
<td>1.30–1.89</td>
<td>$2 \times 10^{-6}$</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>1.10</td>
<td>1.03–1.18</td>
<td>$0.007$</td>
</tr>
<tr>
<td>HbA1c (per%)</td>
<td>2.01</td>
<td>1.58–2.55</td>
<td>$1 \times 10^{-8}$</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.44</td>
<td>1.20–1.73</td>
<td>$0.001$</td>
</tr>
<tr>
<td>HbA1c × duration (%)</td>
<td>0.989</td>
<td>0.981–0.997</td>
<td>$0.007$</td>
</tr>
</tbody>
</table>

Table 5. Summary of the fitted baseline phenotypic model selected to be predictive for each EURAGEDIC population

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Denmark</th>
<th>Finland</th>
<th>France</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio (95% CI)</td>
<td>$P$-value</td>
<td>Odds ratio (95% CI)</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.48 (1.07–2.07)</td>
<td>0.019</td>
<td>1.90 (1.42–2.55)</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>1.28 (1.12–1.45)</td>
<td>&lt;0.001</td>
<td>1.05 (0.94–1.18)</td>
</tr>
<tr>
<td>HbA1c (per%)</td>
<td>4.25 (2.68–6.72)</td>
<td>&lt;0.001</td>
<td>1.67 (1.12–2.49)</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.91 (0.65–1.28)</td>
<td>0.59</td>
<td>1.86 (1.39–2.50)</td>
</tr>
<tr>
<td>HbA1c × duration (%)</td>
<td>0.97 (0.96–0.99)</td>
<td>&lt;0.001</td>
<td>0.99 (0.98–1.01)</td>
</tr>
</tbody>
</table>
The statistical strategy applied in the EURAGEDIC study strives to establish confirmed association given the large number of candidate genes and SNPs that will be tested. Therefore, a two-stage approach was chosen, and all SNP markers showing suggestive evidence of association in the first-line case-control study ($P \leq 0.1$) was to be characterized in the second-line family-based trio study as well. A newly developed method [27] to combine results from the first-line and second-line studies was then used to assess overall evidence for association. One major limitation to the overall study is the relatively small number of trios included, due to unexpected difficulties in the recruitment phase.

A post-hoc power calculation for the first- and second-line studies combined revealed that the EURAGEDIC study has good power ($\sim 85\%$) to detect relatively small genetic differences (GRR = 1.3) provided that the allele is frequent in the population. Another limitation to the EURAGEDIC study is the relatively high OR detectable with the number of patients included. In order to have $85\%$ power to detect a GRR of 1.2, inclusion of about twice the number of cases ($n \sim 2000$) would be necessary.

Population stratification is a source of potential confounding in case-control studies. Ethnic diversity between cases and controls within each country would potentially lead to stratification effects and inflation of the type 1 error. The association for the genomic control markers in the EURAGEDIC was compatible with the expectation of no association (data not shown) indicating that stratification within one or more of the populations is an unlikely source of positive association. Genetic diversity across countries can be dealt with by calculating weighted risks across the three countries; marked genetic diversity is likely to increase the type 2 error. In addition, our collection of trio families provides a valuable resource in which to robustly test potential diabetic nephropathy susceptibility alleles.

Large-scale cohorts of type 1 diabetic patients dedicated to complications are available for genetic studies. The GoKinD cohort, using a very similar strategy, focuses on American diabetic patients [28]. The DCCT/EDIC cohort, also in North America, is focusing on the development of DN, including microalbuminuria, using a follow-up design [29]. Other important cohorts such as the Pittsburgh Epidemiology of Diabetes Complications Study [30] or the EURODIAB complication study [31] also presented the association of DN with genetic markers. In this context, the EURAGEDIC cohort is of peculiar importance due to its large size, its careful phenotypic definition of cases and controls, its family-based and cross-sectional combined approaches and its multinational European recruitment.

In conclusion, the EURAGEDIC study based on a systematic selection and exploration of candidate genes for diabetic complications in large collections of well-characterized Type 1 diabetic patients has the power to identify and validate the contribution of common alleles as genetic risk factors for DN.

### Supplementary Materials

For Supplementary Materials, please refer NDT online.

### Acknowledgements

The EURAGEDIC study was made possible through funding from the European Commission through contract number QLG2-CT-2001-01669. All type 1 diabetes patients and their family are sincerely acknowledged.

The Danish Diabetes Association and the Sehested Hansen Foundation are thanked for their continued support to this study.

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Oulf Pedersen, Steno Diabetes Center, Gentofte, Denmark and Ludovic Drouet, Hôpital Lariboisière, Paris France are kindly acknowledged for providing non-diabetic individuals from population-based studies.

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Conflict of interest statement. None declared.

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