Study of candidate genes affecting the progression of renal disease in autosomal dominant polycystic kidney disease type 1

Bárbara Tazón-Vega1, Mireia Vilardell2, Laureano Pérez-Oller3, Elisabet Ars1, Patricia Ruiz1, Olivier Devuyst4, Xose Lens5, Patricia Fernández-Llama1, José Ballarín1 and Roser Torra1

1Fundació Puigvert. Cartagena 340-350, 08025 Barcelona, Spain, 2Unitat de Genètica. Dept. Ciències Experimental i de la Salut. Universitat Pompeu Fabra. Av. Aiguader 80. 08003 Barcelona, Spain, 3Hospital General de Vic. Francesc Pla “el Vigatà” 1. 08500 Vic, Barcelona, Spain, 4Université Catholique de Louvain Medical School, Avenue Emmanuel Mounier, 54. B - 1200 Bruxelles, Belgium and 5Complexo Hospitalario Universitario de Santiago Gil Casares. Galeras s/n, 17505 Santiago de Compostela, Spain

Abstract
Background. Autosomal dominant polycystic kidney disease (ADPKD) is a systemic disorder with a wide spectrum of renal involvement. Differences in the age at onset of end-stage renal disease (ESRD) are partially explained by the genetic heterogeneity of the disease but intrafamilial variability remains to be explained. Modifier genes may play a role in disease severity.

Methods. A total of 355 PKD1 patients from 131 families belonging to three different European centres were analysed. According to the age at onset of ESRD patients were classified into two groups: early and late onset. Two different cut-offs were used. Based on literature, early onset was firstly considered when ESRD was reached before 40 years of age and late onset after 60 years of age. Secondly, according to the bimodal distribution of age at onset of ESRD in our population we established two groups with similar variability and the cut-offs were assigned before 48 years of age and after 56 years of age. These groups of patients were then analysed by two different complementary perspectives: (i) using ESRD onset as a quantitative trait when performing survival analysis and Cox regression analysis, and (ii) considering it a qualitative trait. The candidate genes (and polymorphisms) studied were the following: NOS3 (T-786C and E298D), BDKRB1 (−699 G > C), BDKRB2 (R14C), TGFB1 (−509 C > T, R25P and L10P), ACE (I/D), EGFR (IVS1CA) and PKD2 (−9780 G > A, −718 A > G and 83 C > G).

Results. The results disclosed that the ACE polymorphism had a slight influence on the age of onset of ESRD in ADPKD patients and the NOS3 and BDKRB1 polymorphisms showed a very slight involvement in renal outcome.

Conclusions. Our results discard the most prominent functional genes suggested to date, to have a major effect on ADPKD progression in this cohort. Genes strongly implicated in disease severity are yet to be identified. The description of such genes would allow us to establish a prognosis for ADPKD and eventually to develop therapeutic interventions.

Keywords: ACE; ADPKD; BDKRB1; BDKRB2; disease progression; EGFR; ESRD; modifier genes; NOS3; PKD1; PKD2; polycystic kidney disease; polymorphisms; tgfβ1

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most commonly inherited conditions in humans [1] accounting for 5–10% of patients on renal replacement therapy. The disease is caused by mutations in either PKD1 or PKD2 genes, with PKD1 mutations underlying over 85% of cases [2]. The proteins encoded by PKD1 and PKD2, called polycystin 1 and polycystin 2, interact with each other in the primary cilia of renal epithelial cells and participate in complex signal transduction pathways, which seem to be involved in mechanic/chemosensory functions and have some role in cell proliferation and maturation [3].

ADPKD is a multisystemic disease characterized by the progressive development and enlargement of multiple fluid-filled cysts in the kidney that may ultimately lead to end-stage renal disease (ESRD). Other manifestations include hepatic cysts, cerebral aneurysms and cardiac valve abnormalities [4]. Hypertension occurs in 50–75% of patients prior to
renal insufficiency and it is thought to accelerate the decline in renal function [4].

One of the most striking facts for nephrologists is the extreme variability in renal disease progression among ADPKD patients. In some individuals, kidney cysts are present in early childhood and progression to ESRD occurs before 40 years of age; whereas in others, renal function remains unimpaired throughout life. Interfamilial variability may be explained by locus heterogeneity, PKD1 mutations leading to an earlier age of onset of ESRD than PKD2 mutations [2,5], allelic heterogeneity (the type and localization of the mutations) [6–8] or environmental factors.

Several authors have reported a significant intrafamilial variability of renal disease which cannot be explained by the previous factors. Dalgaard et al. [9] studied 284 patients and their families and concluded that there was an interfamilial variability related to the age of onset of ESRD. Moreover, Milutinovic et al. [10] in a study of 36 families, disclosed that expression of ADPKD varies widely not only among, but also within families. Likewise, in a study from our group we found an interfamilial variability of twice the intrafamilial variability but the latter was still relevant [11]. This intrafamilial heterogeneity has also been reported for dizygotic twins [12] and affected parent–offspring pairs [11,13,14]. Intrafamilial variability can be attributed to the genetic background, as recently demonstrated for the PKD1 phenotype [15–17]. This putative genetic modifier background might affect either cystogenesis itself or different clinical conditions (such as hypertension) that are associated with disease progression. Studies in animal models of polycystic kidney disease have corroborated that the course of the disease is greatly influenced by modifier genes [18,19].

We use the term ‘modifier gene’ here to denote any gene unlinked to the PKD1 or PKD2 loci whose genotype correlates with the ADPKD phenotype. Dependent on genetic background, animals showed a variable phenotype, and cross-breeding revealed the existence of several modifying loci, which have not yet been found in humans. Comparison of sibling pairs with more distant related family members could give some clues to identify modifier loci. If the phenotypic variation is determined primarily by genes unlinked to the PKD1 or PKD2 loci (modifier genes), the phenotypic correlation will decrease with the degree of relationship, being strongest between pairs of monozygotic twins and decreasing to zero for distant relatives in the same ADPKD family. Although the genome-wide scan for ADPKD has not yet been carried out, many candidate genes have been studied in small groups of patients by several authors.

In the present study, we have used a genetic association strategy to examine the relationship among the clinical outcome of 355 PKD1 patients from three European centres and the polymorphisms of the following candidate modifier genes: endothelial nitric oxide synthase (NOS3), angiotensin-converting enzyme (ACE), tumour growth factor-ß1 (TGFB1), Bradikinin receptor B1 gene (BDKRB1), Bradikinin receptor B2 gene (BDKRB2), epidermal growth factor receptor (EGFR) and polycystic kidney disease type 2 (PKD2). The polymorphisms were chosen in view of their functional effect or biological relevance or their previous association with disease progression in smaller cohorts.

### Patients and methods

#### Patients and controls

A total of 355 ADPKD patients from 131 families were recruited from patients attending three different European centres: Fundació Puigvert, Barcelona, Catalonia (Spain), Hospital Clínic Universitario, Santiago de Compostela, Galicia (Spain) and Université Catholique of Louvain Medical School, Brussels (Belgium) and their related dialysis units. Therefore, three different populations of patients were studied: Catalan (n = 173), Galician (n = 110) and Belgian (n = 72). Diagnosis of ADPKD was considered on the basis of Ravine ultrasound criteria [20]. All patients were Caucasian and suffered from PKD1 disease confirmed by previous linkage analysis performed at each centre. Description of the group of patients is shown in Table 1. Disease progression severity was defined according to the age at onset of ESRD, which was considered the age at which long-term renal replacement therapy was initiated. First, as previously described, [21,22], patients reaching ESRD before 40 years of age were classified into the early onset group (n = 19), and patients in ESRD after 60 years of age or without ESRD and over 60 years of age were classified into the late onset group (n = 33). Second, based on the bimodal distribution of age at onset of ESRD in our population (data not shown) we established two groups with similar variability where patients with an onset of ESRD before 48 years of age were classified into the early onset group (n = 106), and patients with ESRD after 56 years of age or without ESRD and over 56 years of age were classified into the late onset group (n = 50).

The Ethical Committee of each hospital participating in the study approved the procedures and informed consent was obtained from all patients and their families.

Control population consisted of 150 healthy individuals, 50 of them representative of each of the three studied populations.

| Table 1. Description of the 355 PKD1 patients studied and their clinical features |
|---------------------|-----------------|-----------|-----------|
|                     | Catalan | Galician | Belgian | Total |
| Patients            | 173     | 110      | 72       | 355    |
| Families            | 58      | 46       | 27       | 131    |
| Men                 | 73      | 56       | 35       | 164    |
| Women               | 100     | 54       | 37       | 191    |
| ESRD ≤ 40 y         | 67      | 39       | 39       | 145    |
| ESRD ≥ 60 y or no ESRD | 11   | 1        | 7        | 19     |

ESRD, end-stage renal disease; y, years of age.
Gene polymorphisms analysis

Genomic DNA was extracted from peripheral blood samples by the salting-out method [23]. Twelve polymorphisms from seven different genes were analysed. Detailed polymorphism characteristics, PCR primer sequences and product sizes are described in Table 2. All single PCRs were performed in a 15 μl final volume with 50 ng of genomic DNA, 10 pmols of each primer and a 1X final concentration of PCR Master Mix (Promega, Madison, WI). PCR conditions consisted of: initial denaturation at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 30 s at the annealing temperature shown in Table 2, 30 s at 72°C and a final extension at 72°C for 7 min. For the ACE insertion/deletion polymorphism, two PCR rounds were performed. First, the insertion/deletion primer pair was used to detect the ID, II or DD alleles. However, ID heterozygotes can be erroneously considered DD homozygotes because of the preferential amplification of the smaller heterozygotes can be erroneously considered DD homozygous pair was used to detect the ID, II or DD alleles. For the ACE insertion/deletion polymorphism, two PCR rounds were performed. First, the insertion/deletion primer pair was used to detect the ID, II or DD alleles. However, ID heterozygotes can be erroneously considered DD homozygotes because of the preferential amplification of the smaller D allele. Thus, all DD samples were re-amplified in a second PCR round with the insertion-specific primers.

Single nucleotide polymorphism analysis. Ten single nucleotide polymorphisms (SNPs) were analysed applying the single base extension strategy (minisequencing) as shown in Table 3. First, the three SNPs of the PKD2 gene were analysed together, however, the –718 A > G (PKD2) insertion/deletion polymorphism was discarded after having analysed 200 individuals and having detected that all were homozygous for the A allele. Then, the other two SNPs of the PKD2 gene were genotyped separately. Genotyping was carried out according to a specific adaptation of the SNaPshot multiplex system protocol (Applied Biosystems, Foster City, CA). First, PCR products of the PKD2 polymorphisms were pooled as follows: 1.5 μl of –9780G > A and 1.5 μl of R28P. Second, PCR products of the rest of the SNPs were pooled as follows: 4 μl of –786T > C (NOS3), 0.5 μl of R14C (BDKRB2), 1 μl of E298D (NOS3), 1 μl of –509C > T (TGFBI), 6 μl of R25P/L10P (TGFBI) coamplification and 0.5 μl of –699G > C (BDKRB1). Then, pooled PCR products were cleaned up of excess PCR primers and unincorporated dNTPs with 2 μl of ExoSAP-IT (USB, Cleveland, OH) following manufacturer’s instructions. Minisequencing reactions using the two different PCR product pools were performed in a 5 μl final volume, comprising 2.5 μl of SNaPshot ready reaction mix (Applied Biosystems, Foster City, CA), 1.5 μl of cleaned PCR product pool and 0.5 μl of premixed base extension primers giving an individual final concentration that is shown in Table 3. Cycling conditions were performed according to the manufacturer’s protocol. A volume of 0.5 μl from a 1:20 dilution of the multiplex single base extension reaction product was run on an ABI PRISM 3100-Avant genetic analyser (Applied Biosystems, Foster City, CA). Analysis of electropherograms was performed using the GeneScan software version 3.7 and determination of the size of the fragments was based on GeneScan-120 LIZ size standard (Applied Biosystems, Foster City, CA) as shown in Figure 1.

Insertion/deletion and CA repeat analysis. I/D (ACE) and IVS1CA (EGFR) polymorphisms were genotyped separately. ACE PCR products were run on a 2% agarose gel and results are shown in Figure 1. One of the primers of the EGFR CA repeat was fluorescently labelled, thus, its PCR products were run on an ABI PRISM 3100-Avant genetic analyser (Applied Biosystems, Foster City, CA) and the three more frequent genotypes are shown in Figure 1.

Statistical methods

All statistical analysis was performed using R-platform and SPSS software version 11.0. Multiple testing effects were corrected by Benjamini–Hochberg (BH) method, when necessary. Statistical significance was always considered when the P-values < 0.05.

Hardy–Weinberg equilibrium and population homogeneity

The exact Hardy–Weinberg equilibrium test was calculated for the two alleles of each polymorphism. For the IVS1CA (EGFR) polymorphism, the chi-square test was calculated using resampling with a 10 000 resample size instead of the reference distribution [24]. Hardy–Weinberg equilibrium was performed for controls and patients. Homogeneity between controls and patients was assessed with chi-square test using resampling with a 10 000 resample size instead of the reference chi-square distribution (to overcome the limitations of a reduced sample size). Besides, homogeneity between males and females and the three population origins was assessed with the same chi-square tests.

Patient sample validation (one-way ANOVA)

One of the most important limitations in our study is the fact that patients (in some cases) were related. Family independence for the age at onset of ESRD was evaluated performing a variance analysis in which the outcome variable was the age at onset of ESRD and the random effect factor was the family to whom the patient belonged. Only families with more than two affected individuals who had already reached ESRD were selected for analysis using this test. A P-value for the F-value was calculated.

SNP analysis

Qualitative and quantitative analysis. Two analyses were performed: (i) a qualitative analysis between patients with early and late ESRD onset using chi-square tests (one classification used cut-offs < 40 and > 60 years of age and a second classification used cut-offs < 48 and > 56 years of age). All comparisons between groups were performed by chi-square tests using resampling with a 10 000 resample size instead of the reference chi-square distribution. Comparisons were performed for genotypes and alleles [25]. (ii) Quantitative analysis using all the population of the study regardless of ESRD.

Survival analysis. The Kaplan–Meier method was performed using the Wilcoxon–Gehan statistic to compare groups instead of the classical log-rank statistic due to the presence of a high number of censored individuals (young patients that had not reached ESRD at the time of the study). Survival was examined for genotypes and alleles. Cox hazard proportional risk was also estimated.
## Table 2. Characteristics of the nine polymorphisms analysed in this study and their PCR conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Polymorphism</th>
<th>Type</th>
<th>Location</th>
<th>Substitution</th>
<th>PCR primer sequences</th>
<th>Ann. temp</th>
<th>Size</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| NOS3   | CR 7     | −786T > C    | SNP   | Promoter | T > C       | F: GCATGCACCTCTGGCCTGAAGTG  
R: CAGGAAAGCTGCTTCCAGTGCA  
F: AAGGCAAAGACAGTGGGATGGA  
R: CCAGTCAAATCTCTTGGTGCTCA  
F: AGGGAACAACAAGCAAAAGGC  
R: CCAACTGGAAGGCCCCCTTAC  
F: CCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 60°C      | 223 bp | 60    |
| NOS3   | CR 7     | E298D        | SNP   | Exon 7   | G > T       | F: CCACTGGAAGGCCCCCTTAC  
R: AGGGAACAACAAGCAAAAGGC  
F: CCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 62°C      | 248 bp | 61    |
| BDKRB1 | CR 14    | −699 G > C   | SNP   | Promoter | G > C       | F: CCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 58°C      | 191 bp | 27    |
| BDKRB2 | CR 14    | R14C         | SNP   | Exon 2   | C > T       | F: CCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 58°C      | 184 bp | 62    |
| TGFβ1  | CR 19    | −509C > T    | SNP   | Promoter | C > T       | F: CCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 60°C      | 455 bp | 63    |
| TGFβ1  | CR 19    | L10P`       | SNP   | Exon 1   | C > T       | F: TCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 60°C      | 500 bp | 63    |
| ACE    | CR 17    | I/D ins/del  | PCR   | Intron 16|             | F: TCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 64°C      | ins: 490 bp; del: 335 bp | 54    |
| EGF    | CR 7     | IVS1CA      | CA repeat | Intron 1 |              | F: TCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 67°C      | 114–132 bp | GDB |
| PKD2   | CR 4     | −9780G > A   | SNP   | Promoter | G > A       | F: TCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 50°C      | 307 bp | NCBI  |
| PKD2   | CR 4     | −718A > G    | SNP   | Promoter | A > G       | F: TCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 58°C      | 390 bp | NCBI  |
| PKD2   | CR 4     | R28P         | SNP   | Exon 1   | G > C       | F: TCAATTTCCTCCTCCCTGCTCGAG  
R: GGTGGGCGAGCAGTCTCTCCTC | 60°C      | 668 bp | NCBI  |

CR, Chromosome; SNP, single nucleotide polymorphism; F, forward; R, reverse.
*L10P and R25P polymorphisms are coamplified; ins, insertion; del, deletion; ANN. TEMP, annealing temperature; bp, base pair; REF, reference; GDB, Human Genome Database (www.gdb.org); NCBI, National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).
Table 3. Single base extension (minisequencing) primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Minisequencing tailed* primer (5' to 3')</th>
<th>Length</th>
<th>Concentration in reac. mix</th>
<th>Strand</th>
<th>Real substitution</th>
<th>Detected substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS3</td>
<td>−786T&gt;C</td>
<td>GCATCAAGCTTCTCCTCCCTGGC</td>
<td>20nt</td>
<td>0.4µM</td>
<td>Forward</td>
<td>T&gt;C</td>
<td>T&gt;C</td>
</tr>
<tr>
<td>BDKRB2</td>
<td>R14C</td>
<td>tatataGTGGGACGGAGTCTCTCT</td>
<td>25nt</td>
<td>0.05µM</td>
<td>Reverse</td>
<td>C&gt;T</td>
<td>G&gt;A</td>
</tr>
<tr>
<td>NOS3</td>
<td>E298D</td>
<td>atatatatatGTCAGCCCAGATGA</td>
<td>29nt</td>
<td>0.1µM</td>
<td>Forward</td>
<td>G&gt;T</td>
<td>G&gt;A</td>
</tr>
<tr>
<td>TGFBI</td>
<td>−593G&gt;T</td>
<td>atatatatatatGGAACAGGAAC</td>
<td>33nt</td>
<td>0.1µM</td>
<td>Reverse</td>
<td>C&gt;T</td>
<td>G&gt;A</td>
</tr>
<tr>
<td>TGFB1</td>
<td>R25P</td>
<td>atatatatatatataatGTTGGGCACGGAGTCCTCT</td>
<td>37nt</td>
<td>0.4µM</td>
<td>Forward</td>
<td>G&gt;C</td>
<td>G&gt;C</td>
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<tr>
<td>TGFB1</td>
<td>L10P</td>
<td>atatatatatatatatatatatatatGTCAGCCAGTGA</td>
<td>41nt</td>
<td>0.4µM</td>
<td>Reverse</td>
<td>C&gt;T</td>
<td>G&gt;A</td>
</tr>
<tr>
<td>BDKRB1</td>
<td>−699G&gt;C</td>
<td>atatatatatatataatGATGAAATGTTCT</td>
<td>45nt</td>
<td>0.05µM</td>
<td>Forward</td>
<td>G&gt;C</td>
<td>G&gt;C</td>
</tr>
<tr>
<td>PKD2</td>
<td>−9780G&gt;A</td>
<td>CACAGTAGCTCACTATGAC</td>
<td>20nt</td>
<td>0.6µM</td>
<td>Forward</td>
<td>G&gt;A</td>
<td>A&gt;G</td>
</tr>
<tr>
<td>PKD2</td>
<td>−718A&gt;G</td>
<td>atatatatatatatatatatatatatatatGATGAGTA</td>
<td>26nt</td>
<td>0.2µM</td>
<td>Forward</td>
<td>A&gt;G</td>
<td>A&gt;G</td>
</tr>
<tr>
<td>PKD2</td>
<td>R28P</td>
<td>atatatatatatatatatatatatatatatatataatGAGATGACA</td>
<td>31nt</td>
<td>5µM</td>
<td>Reverse</td>
<td>G&gt;C</td>
<td>G&gt;C</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism; *primer tails are printed in lower case; nt, nucleotides; REAC, reaction.

Results

Before studying the correlation of the polymorphisms with the age onset of ESRD, we analysed the homogeneity of allele frequencies between controls and patients, males and females (raw). No association was found (raw, BH corrected P = 0.048). However, using the Haplo.stats library from R-platform, an association was found between the alleles of the two SNPs analysed in the TGFBI gene (−593G>T, L10P), raw P = 0.016 and BH corrected P = 0.045. The influence of environmental factors, the family to whom the patient belonged, and the age at onset of ESRD and the family to whom the patient belonged, was accounted for (raw, BH corrected P = 0.016). A descriptive analysis was performed calculating haplotypes to investigate a possible influence on ESRD. The observed distribution of alleles confirmed the Hardy-Weinberg equilibrium except for the ESRD onset. A descriptive analysis is performed calculating haplotypes using haplo.stats library from R-platform.

ANOVA test strongly reinforces the accuracy of performing the haplotypes analysis. To study these environmental factors, one-way ANOVA test was performed on 80 patients who had reached ESRD belonging to 33 families (only families considered for this purpose). No association was found (raw, BH corrected P = 0.165). To study the influence of genetic factors, residual variability was verified. Familial effect only explains 25.8% of model variability. Moreover, the consequences of this method were not considered in the analysis. No association was found (raw, BH corrected P = 0.165). To study the influence of environmental factors, one-way ANOVA test was performed on 80 patients who had reached ESRD belonging to 33 families (only families considered for this purpose). No association was found (raw, BH corrected P = 0.165). The observed distribution of alleles confirmed the Hardy-Weinberg equilibrium except for the ESRD onset. A descriptive analysis is performed calculating haplotypes using haplo.stats library from R-platform.

Genotypes from the different genes were combined into three SNPs (NOS3, TGFBI, TGFB1) and three genotypes (NOS3, TGFBI, TGFB1) were analysed taking into account that the two SNPs analysed in the TGFBI gene (−593G>T, L10P, raw P = 0.016 and BH corrected P = 0.045). The observed distribution of alleles confirmed the Hardy-Weinberg equilibrium except for the ESRD onset. A descriptive analysis is performed calculating haplotypes using haplo.stats library from R-platform.
in the severity of the *PKD1* disease, but it is not attributable to the frequencies of polymorphisms in the general population. Therefore, we confirmed that the three populations can be analysed as a whole to study the correlation of the genotypes of polymorphisms with the age at onset of ESRD.

**Renal survival analysis**

We performed a survival analysis according to sex, population, genotypes and of polymorphism alleles. There were no significant differences for any of these factors except for the R25P (*TGFB1*) genotype (raw and BH corrected *P* < 0.001), however, we did not take this result into account due to the fact that for this polymorphism there is one category with a single individual which distorts the test (data not shown). Cox regression confirms these results (data not shown).

To study the correlation of the genotypes of polymorphisms with the age at onset of ESRD, we performed a chi-square test between the early ESRD onset group (ESRD < 40 years of age, mean 64 ± 4.5) and the late ESRD onset group (ESRD > 60 years of age, mean 37 ± 3.5) (Table 4). Significant differences were found in the frequencies of the I/D (*ACE*) genotypes (raw *P* = 0.0379, BH corrected *P* = 0.304). Moreover, *P*-values < 0.2 were found for the −509C>T (*TGFB1*) and L10P (*TGFB1*) polymorphisms (raw *P* = 0.1572, BH corrected *P* = 0.329 and raw *P* = 0.1913, BH corrected *P* = 0.329, respectively) and between males and females (raw *P* = 0.0982, BH corrected *P* = 0.329). No statistically significant different results were achieved when testing the correlation between polymorphisms and onset of ESRD in groups classified according to our population bimodal distribution of age at onset of ESRD (<48 and >56 years of age).

Haplotype analysis, including all analysed SNP, for the different ACE polymorphism genotypes was performed with the limitation of sample size. No differences were observed between the early onset group and the late onset group (data not shown).
Discussion

The study of phenotypic variability in inherited disorders has been the purpose of many studies for a long time, to better understand the pathogenesis of the disease, to establish a more precise prognosis and to improve genetic counselling. The intrafamilial variability in such disorders rules out locus and allelic heterogeneity and only leaves room for a slight environmental factor and a supposed strong modifier gene action. Therefore, the role of candidate modifier genes has been widely studied in many inherited disorders. For most of these studies there are two main problems: genotyping families when the disease shows locus heterogeneity and having accurate clinical traits to determine disease severity. For the latter, as regards ADPKD, it seems most appropriate to use the age of onset of ESRD as an objective endpoint. On the other hand, genotyping these patients is cumbersome and, therefore, absent in most studies carried out to date. In the present study, we analyse the effect of some candidate modifier genes in a large PKD1 population.

Thanks to heritability studies, we now have an estimation of the proportion of the total phenotypic variability in PKD1 that can be attributed to variation in modifier genes. Fain et al. [15] obtain a heritability between 43 and 50% regarding the age of onset of ESRD and Paterson et al. [16] found a heritability of 78%. Moreover, Persu et al. [17] found a significant excess of variability in renal disease progression in ADPKD siblings when compared with monozygotic twins.

Table 4. Comparison (chi-square test) of genotypes of the early ESRD onset group and the late ESRD onset group

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotypes</th>
<th>Global results</th>
<th>Late ESRD onset</th>
<th>Early ESRD onset</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Frequency</td>
<td>%</td>
<td>Frequency</td>
<td>%</td>
</tr>
<tr>
<td>−786T &gt; C (NOS3)</td>
<td>CC</td>
<td>10</td>
<td>19%</td>
<td>7</td>
<td>21%</td>
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<tr>
<td></td>
<td>TC</td>
<td>26</td>
<td>50%</td>
<td>18</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>16</td>
<td>31%</td>
<td>8</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>52</td>
<td></td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>R14C (BDKRB2)</td>
<td>CC</td>
<td>48</td>
<td>92%</td>
<td>30</td>
<td>91%</td>
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<td>Total</td>
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Significance was established for P-values <0.05.
In bold: significant.
*Significance <0.2.
Apart from these population-based studies, several authors have attempted to demonstrate a causative relationship between some candidate modifier genes and ADPKD phenotype. However, these studies yielded divergent results, which can be explained by small sample size, population admixture, lack of controls and, mainly, lack of PKD1/PKD2 genotyping. The present study comprises the largest number of genotyped patients to date. In this study, we have analysed seven different candidate genes which may play different roles, exposed herein, in ADPKD.

Recently, the role of the Kallikrein-kinin system in the inflammatory process in the kidney has been stressed, and a protective effect of kinin peptides against the development of renal disease [26] has been suggested. Moreover, Zychma et al. [27] supported, as well as Bachvarov et al. [28], the hypothesis of the protective role of the BDKRB1 and BDKRB2 polymorphisms in the development of ESRD. Essentially, an inflammatory mechanism contributes to kidney damage in ADPKD; therefore, we decided to investigate whether polymorphisms in the BDKRB1 and BDKRB2 genes (−699 G > C and R14C, respectively) were involved in disease progression in ADPKD. We did not find any genotype-phenotype correlation for these polymorphisms.

Several studies have shown that a decrease of nitric oxide (NO) production may be important in the progression of renal disease [29,30], and a significant endothelial dysfunction has been described in ADPKD patients [31]. Nakayama et al. [32] described that the −786T > C SNP, in the promoter of the NOS3 gene, is a functional polymorphism, the C allele being associated with higher gene expression. Moreover, the D allele of the E298D polymorphism, also in the NOS3 gene, is known to lower the enzyme activity in association with post-translational modifications [33].

A significant correlation between polymorphisms in the NOS3 gene and ADPKD disease progression has been detected in some studies [34,35]. In one study [33], the association was confirmed in a subgroup of male PKD1 patients and it was reflected by a molecular counterpart in terms of eNOS activity and processing [33,36]. In contrast, the association was not detected in another series [37]. Only a slight correlation (not significant) between the promoter polymorphism of NOS3 and renal survival was found in our multicentric PKD1 population. These discordant results may originate from different inclusion strategies, gender and/or population admixture and genetic ascertainment [38]. In addition, the allelic frequency of the NOS3 variant is different among the three populations studied.

After discovering that EGFR is an important mitogen for epithelial tubular cells, and taking into account that tubular cell mitosis is a trait of ADPKD kidneys, some authors have reported alterations of EGF/EGFR axis in primary cell cultures from ADPKD patients and PKD mice that could lead to tubular cell proliferation [39,40]. Moreover, some investigations point to a therapeutic use of EGFR tyrosine kinase inhibition to slow the process of cystogenesis [41,42]. Magistroni et al. [43] have studied the effect of the EGFR microsatellite polymorphism on the age at onset of ESRD in 46 ADPKD patients with ESRD. Their results suggest there might be a slight genotype–phenotype correlation, however, the small size of the sample and the fact of not knowing the gene status of the ADPKD patients make the results questionable. In the present study, we did not find any genotype–phenotype correlation regarding the EGFR microsatellite. However, the statistic analysis was quite limiting due to the presence of 10 different alleles of the CA repeat in the studied population. Very large populations should be studied when analysing CA repeats with more than three alleles.

**TGFβ1** is a cytokine from the family of the dimeric polypeptidic growth factors. Almost every cell in the organism (epithelial, endothelial, haematopoietic, neuronal and connective tissue) produces and has a receptor for this growth factor. **TGFβ1** controls cellular proliferation and differentiation, embryological development, immunological system, wound healing and angiogenesis. One of the most relevant functions of **TGFβ1** is its involvement in tissue fibrosis when over-expressed. An increase of **TGFβ1** may cause an over-production of extracellular matrix leading to fibrosis. This mechanism has been implicated in the progression of renal failure, especially in glomerular nephropathies and chronic allograft nephropathy [44,45]. Moreover, a fibrogenic role of angiotensin II mediated by **TGFβ1**, has been suggested [46]. For example, drugs inhibiting the renin–angiotensin axis diminish **TGFβ1** expression, stopping the development of renal fibrosis [44,47]. Some polymorphisms of **TGFβ1**, such as G915C, C509T or T869C influence the blood levels of **TGFβ1**. Also, some genotypes of these polymorphisms have been implicated in several nephropathies. It has been proposed that renal failure in ADPKD is mainly the consequence of interstitial changes such as fibrosis and tubular atrophy. Therefore, **TGFβ1** may play a role in disease progression in ADPKD. Lee et al. [48] studied the influence of these polymorphisms in a small, non-genotyped ADPKD population and found no effect. In our study, we only found a slight non-significant correlation between L10P (**TGFβ1**) and renal survival. Both results point to a low effect of this gene on ADPKD progression.

The description by Rigat et al. [49] of a polymorphism in the **ACE** gene, and the fact that the D allele is associated with higher plasma levels of ACE and therefore an increased production of angiotensin II has been followed by several studies focused on the relationship between this polymorphism and diseases where the renin–angiotensin system (RAS) may play a pathogenic role. Initial reports suggested an association between D allele and cardiopathy, mainly with ischaemic heart disease and left ventricular hypertrophy. Subsequently, other processes have been studied, including renal diseases with progression to chronic renal failure. In the same direction, some authors have demonstrated the association between the **ACE** gene polymorphism and nephropathy, the D allele being...
deleterious in the progression of diabetic nephropathy [50], Ig A nephropathy [51] and hypertensive nephropathy [52]. On the other hand, it has become increasingly apparent that the RAS plays an important role in cystogenesis in ADPKD. Angiotensin II has been demonstrated to be a growth factor in renal cell systems and it has also been proved that renal cystogenesis induced experimentally is enhanced by conditions that activate RAS and is lessened by suppression of RAS [53]. Based on these findings, it has been proposed that RAS may modulate cyst formation, probably due to altered sensitivity of tubular epithelial cells to angiotensin II. Some authors have suggested that abnormal distribution of renin-containing cells in ADPKD could affect the intrarenal action of renin and its access to the circulation. On the other hand, it is accepted that cyst fluid contains renin with enzymatic activity; in fact, it seems that tubulocystic epithelium has the potential to synthesize renin because cyst-derived epithelial cells in culture express renin mRNA [54]. Also, several studies have demonstrated a relationship between increased RAS activity and hypertension in ADPKD patients [55]. All these features have led to several studies devoted to determine the significance of this association. Only four studies have found a certain relationship [22,34,35,56]. The rest of the studies that have been performed were not able to demonstrate a significant relationship between the ACE polymorphism and renal survival in ADPKD [21,57–60]. As a matter of fact, the present study, which represents by far the largest study of this type carried out to date, only discloses a slightly worse prognosis for those patients carrying the D allele. Although the results are only significant for the comparison between the early ESRD onset group and the late ESRD onset group, the survival analysis already shows a tendency towards a better survival for those patients not carrying the D allele. These results point to a deleterious effect of the DD genotype on the PKD1 prognosis but discard the ACE gene as a principal modifier gene.

Finally, PKD2 may also be considered a potential modifier gene for PKD1. Polycystin-1 and -2 form heteromeric complexes through a coiled coil region in the intracellular domain of polycystin-1 that binds to the last 97 amino acids in polycystin-2 [61]. Polycystin-1 and -2 also colocalize in the primary cilium of renal epithelial cells. Recent studies proposed a role of the primary cilium as a mechanoreceptor that may sense changes in apical fluid flow and may be able to transduce them into an intracellular Ca$^{2+}$ signalling response [62]. This model involves the participation of polycystin-1 as a mechanical sensor of ciliary bending induced by luminal fluid flow. Bending of the cilium would cause a conformational change in polycystin-1 that would in turn activate polycystin-2-associated Ca$^{2+}$ channel. Ca$^{2+}$ influx would subsequently stimulate intracellular ryanodine-sensitive stores to release Ca$^{2+}$ to the cytosol, thus increasing the intracellular Ca$^{2+}$ concentration. The resulting increase in cytosolic Ca$^{2+}$ would then trigger intracellular signalling pathways leading to normal kidney development. Considering that both polycystins interact and belong to the same complex it seems most feasible that slight conformational changes produced by a polymorphism in PKD2 may influence PKD1 and the polycystin complex in general. Therefore, we studied three polymorphisms in the PKD2 gene and promoter. We chose −9780 G > A, −718 A > G for their localization in the promoter region, with more probabilities of being involved in gene regulation. However −718 A > G was not informative at all, and analysis of −9780 G > A yielded no significant results regarding severity of PKD1 disease. We also analysed 83G > R (R28P), previously described by our group [63] and being a very informative polymorphism. Unfortunately, this PKD2 polymorphism did not seem to exert any influence on the age of onset of ESRD in PKD1 patients. Although the PKD2 polymorphisms studied in this article do not seem to be relevant for PKD1 outcome, the PKD2 gene is the only gene whose interaction with PKD1 has been proven and, therefore, is the most convincing candidate to influence PKD1 outcome.

The present study is the largest to date, with all patients being genotyped for the PKD genes. However, we dealt with some limitations, such as the fact that we worked with some related individuals and the presence of many young patients who had not reached ESRD at the time of the study, which weakened the statistical analysis. On the other hand, our population was also heterogeneous, originating from three very distinct geographic areas, as attested by different allelic frequencies.

Nevertheless, our results in this large PKD1 population may be summarized in the conclusion that none of the polymorphisms studied plays a major role in disease progression in PKD1, in spite of some of them being functional and having been demonstrated to play a role in renal survival in ADPKD. However, the negative nature of this study does not exclude the possibility that the candidate genes being tested may contain variants of moderate effect size that influence renal disease severity in PKD1.

Our results support the hypothesis of genetic background being the main cause of intrafamilial variability in ADPKD but do not suggest that the most prominent genes suggested to date, have a major effect on disease progression. Unless the increased knowledge on polycystins discloses new candidate modifier genes for ADPKD, the most feasible approach seems to be the genome wide-scan in PKD1 sibling pairs. The finding of such genes would be of utmost relevance in terms of assessing disease prognosis and for future therapeutic approaches.

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References


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