Modifier effect of ENOS in autosomal dominant polycystic kidney disease


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Received September 10, 2001; Revised and Accepted November 29, 2001

A significant phenotypical variability is observed in autosomal dominant polycystic kidney disease (ADPKD). ADPKD is associated with altered endothelial-dependent vasodilation and decreased vascular production of nitric oxide (NO). Thus, ENOS, the gene coding for the endothelial nitric oxide synthase (eNOS), could have a modifier effect in ADPKD. In order to test this hypothesis, we genotyped 173 unrelated ADPKD patients from Belgium and the north of France for the Glu298Asp, intron 4 VNTR and T-786C polymorphisms of ENOS and looked for their influence on the age at end-stage renal disease (ESRD). In males (n = 93), the Glu298Asp polymorphism was associated with a lower age at ESRD (Glu/Asp + Asp/Asp: 49.0 ± 1.2 years, n = 53; Glu/Glu: 53.5 ± 1.5 years, n = 40; simple regression, P = 0.02; multiple regression, P = 0.006). This effect was confirmed in a subset of males linked to PKD1 and reaching ESRD before age 45, and by a cumulative renal survival analysis in PKD1-linked families. Further studies demonstrated that NO synthase (NOS) activity was decreased in renal artery samples from ADPKD males harbouring the Asp298 allele, in association with post-translational modifications and partial cleavage of eNOS. No significant effect of the other polymorphisms was found in males, and no polymorphism influenced the age at ESRD in females. In conclusion, the frequent Glu298Asp polymorphism of ENOS is associated with a 5 year lower mean age at ESRD in this subset of ADPKD males. This effect could be due to a decreased NOS activity and a partial cleavage of eNOS, leading to a further decrease in the vascular production of NO.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common monogenic hereditary diseases (prevalence: 1:400–1:1000). It is characterized by the development of multiple cysts in both kidneys. These cysts grow slowly over decades, until renal failure occurs. By the age of 60 years, about half the patients with ADPKD have progressed to end-stage renal disease (ESRD). In Europe and North America, ADPKD is responsible for 10% of the patients requiring renal replacement therapy (1).

Genetic and molecular studies have revealed that ADPKD is due to mutations in two major genes, PKD1, responsible for ~85% of cases, and PKD2, which accounts for the vast majority of the remaining cases (1). The proteins encoded by PKD1 and PKD2 (polycystins 1 and 2, respectively) probably interact in the plasma membrane to participate in a signal transduction pathway controlling renal tubular cell maturation (2).

One of the most striking features of ADPKD is the substantial variability in the severity of renal phenotype, primarily assessed by the age at ESRD. This variability is observed among families, family members and even dizygotic twins (3,4). Interfamilial phenotypic variability may be explained by distinct effects of mutations in PKD1 and PKD2 and also by the nature of the mutation itself. Indeed, PKD2 is clinically milder than PKD1 disease, as witnessed by a later age at ESRD and a lower prevalence of hypertension (5). In addition, different mutations in the same gene are associated with differences in the mean age at ESRD in both PKD1 (5) and PKD2 families (6). Intrafamilial phenotypic variability could be explained by at least two mechanisms: the second hit event and the effect of modifier genes. If cyst formation is triggered by a second hit, i.e. a somatic mutation in the allele unaffected by germline mutation (7), micro-environmental or genetic factors determining the rate of second hit could be a valuable explanation. Alternatively, modifier genes could exert either a...
Intron 4 VNTR: not significantly different according to gender (Table 1) and Caucasian populations. The genotype frequencies were close to those previously described in P230. The observed genotype frequencies did not deviate from the expected frequencies (Fig. 1) is shown in Table 1. For each polymorphism, the distribution of the three diallelic polymorphisms of ENOS in ADPKD would be expected to be more critical in males than in premenopausal women. Indeed, both endothelial-dependent vasodilatation (15,16) and total body production of NO (17) are decreased in men, which arguably renders them more sensitive than women to small modifications of NO production.

In order to investigate a potential modifier effect of ENOS in ADPKD, we assessed the influence of the three most studied polymorphisms of ENOS (Glu298Asp, intron 4 VNTR, T-786C) on the age at ESRD in males and females from a large series of unrelated ADPKD patients recruited in Belgium and the north of France. We further analysed the NOS activity and the expression of eNOS at the mRNA and protein levels in renal artery samples from ADPKD patients according to genotype at the Glu298Asp locus.

RESULTS

Characteristics of the ADPKD population studied

A total of 182 unrelated patients at ESRD were recruited in three academic centres in Belgium and France. From 57 potentially informative families, linkage to PKD1 and PKD2 was established in 27 and nine families, respectively. Patients belonging to PKD2 families were excluded from the study population, which therefore consisted of 173 unrelated patients (93 males and 80 females). Mean age at ESRD was not significantly different in the three centres (Saint Luc Academic Hospital, Brussels: 50.5 ± 1.0, n = 102; U.Z. Gasthuisberg, Leuven: 55.0 ± 1.8, n = 35; Necker Hospital, Paris: 51.3 ± 1.6, n = 36, P = 0.1) and was not significantly different in males and females (Table 1).

Distribution of ENOS polymorphisms and linkage disequilibrium

The distribution of the three diallelic polymorphisms of ENOS (Fig. 1) is shown in Table 1. For each polymorphism, the observed genotype frequencies did not deviate from the Hardy-Weinberg equilibrium (Glu298Asp: \( \chi^2_{1df} = 0.3, P = 0.7 \); intron 4 VNTR: \( \chi^2_{1df} = 0.01, P = 1; \) T-786C: \( \chi^2_{1df} = 0.7, P = 0.3 \) and were similar to those previously described in Caucasian populations (18). The genotype frequencies were not significantly different according to gender (Table 1) and centre (P = 0.3, 0.1 and 0.9 for the Glu298Asp, intron 4 VNTR and T-786 polymorphisms, respectively). The three polymorphisms were in significant linkage disequilibrium (P < 0.001). The a allele of intron 4 VNTR was strongly associated with the Glu allele of the Glu298Asp polymorphism and the C allele of the T-786C polymorphism. In contrast, the Glu298Asp and T-786C polymorphisms were in only weak to moderate linkage disequilibrium (Table 2).

| Table 1. Age at ESRD and distribution of the genotypes of ENOS polymorphisms in male and female ADPKD subsets |
|---|---|---|---|---|
| Total (n = 173) | Male (n = 93) | Female (n = 80) | P-value |
| Age at ESRD (years) | 51.6 ± 0.8 | 50.9 ± 1.0 | 52.3 ± 1.3 | 0.4a |
| Glu298Asp | | | | |
| Glu/Glu | 81 (46.8%) | 40 (43.0%) | 41 (51.2%) | |
| Glu/Asp | 77 (44.5%) | 45 (48.4%) | 32 (40.0%) | |
| Asp/Asp | 15 (8.7%) | 8 (8.6%) | 7 (8.8%) | |
| Intron 4 VNTR | | | | |
| bb | 123 (71.1%) | 64 (68.8%) | 59 (73.8%) | |
| ba | 46 (26.6%) | 27 (29.0%) | 19 (23.8%) | |
| aa | 4 (2.3%) | 2 (2.2%) | 2 (2.4%) | 0.7b |
| T-786C | | | | |
| TT | 61 (35.3%) | 27 (29.0%) | 34 (42.5%) | |
| CT | 89 (51.4%) | 53 (57.0%) | 36 (45.0%) | |
| CC | 23 (13.3%) | 13 (14.0%) | 10 (12.5%) | 0.2b |

The influence of gender was assessed using Student’s t-test (age at ESRD) or \( \chi^2 \) test (genotype frequencies). Values are expressed as means ± SEM.

\[ \text{ENOS (7q35)} \]

\[ \text{T-786C} \]

\[ \text{Intron 4 VNTR} \]

\[ \text{Glu 298 Asp} \]

\[ \text{C CT TT CC} \]

\[ \text{C bb ba aa} \]

\[ \text{C GG GT TT} \]
Influence of ENOS polymorphisms on the age at ESRD in ADPKD

Male patients with ADPKD harbouring the Asp allele of the Glu298Asp polymorphism had a significant, 4.5 year lower mean age at ESRD than Glu/Glu patients (Table 3). The age at ESRD was increased, though less significantly, in patients harbouring the a allele of intron 4 VNTR, as compared to patients with the bb genotype. The T-786C polymorphism had no effect on the age at ESRD in males (Table 3). The distribution of the Glu298Asp polymorphism was significantly different when ADPKD male patients were distinguished according to age at ESRD, with 50 years of age being the cut-off. Patients harbouring the (Glu/Asp + Asp/Asp) genotypes were significantly overrepresented (67%, 31/46) in the subset of patients reaching ESRD before age 50, compared to patients at ESRD after age 50 (47%, 22/47). In female ADPKD patients, none of the polymorphisms of ENOS had an effect on the age at ESRD (Table 3).

The effect of the Glu298Asp polymorphism on the age at ESRD in the male subset was confirmed by multiple regression analysis in models including either intron 4 VNTR (P = 0.025, r² = 0.08) or T-786C (P = 0.006, r² = 0.13) polymorphisms. The model including Glu298Asp and T-786C, the two polymorphisms in weak to moderate linkage disequilibrium, explained a higher proportion of the variance (r² = 0.2) and was thus retained as the best fit model. It yielded a P-value of 0.006 for combined (Glu/Asp + Asp/Asp) versus Glu/Glu genotypes across the three genotypes of T-786C (Table 4). In contrast, multiple regression analysis did not confirm the effect of intron 4 VNTR in models including either of the two other polymorphisms. These findings were confirmed by haplotype analysis for the Glu298Asp and T-786C polymorphisms (P = 0.02, data not shown).

Of note, the effect of the Glu298Asp polymorphism on the age at ESRD was mainly due to Glu/Asp patients (48.5 ± 1.3 years, n = 45). The mean age at ESRD of the eight patients harbouring the Asp298 allele at the homozygous state was 52.1 ± 2.9 years. Two of them reached ESRD at age 61 and 67 years: since they belong to non-informative families, it was impossible to rule out PKD2 linkage in these two particular patients. The mean age at ESRD in the remaining six Asp/Asp patients was 48.2 ± 1.7 years, similar to that found in the Glu/Asp subgroup. The mean age at ESRD in the Glu/Glu patients (53.5 ± 1.5 years, n = 40) was similar to that reported previously for PKD1 families in a similar population (6).

The effect of the Glu298Asp polymorphism was also investigated in the subset of male patients belonging to PKD1-linked families. In this subset (n = 17), the Glu/Asp and Asp/Asp genotypes were associated with a 8 year lower age at ESRD (45.8 ± 2.8 versus 54.1 ± 3.1 years, P = 0.067). Because previous studies in similar ethnic groups showed that the prevalence of PKD2 mutations is negligible in ADPKD patients reaching ESRD before the age of 45 years (1,4,6), we performed the same analysis for the subset of male patients linked to PKD1 and those with ESRD before the age of 45 years. In this subset (n = 37), the Glu/Asp and Asp/Asp genotypes were associated with a 6 year lower age at ESRD (42.4 ± 1.3 versus 48.5 ± 2.6 years, P = 0.03). As in the whole population, the effect of the Glu298Asp polymorphism was not observed in the subsets of female patients linked to PKD1.

It should be noted that the distribution of the Glu298Asp genotypes was not significantly different in the whole ADPKD population studied and in the subgroups including PKD1-linked patients.

In addition to the analyses primarily performed on unrelated ADPKD patients at ESRD, 38 affected male patients with (n = 19) or without renal failure (n = 19) belonging to the 27 PKD1-linked ADPKD families were included in a Kaplan–Meier cumulative renal survival analysis (Fig. 2). Although performed on a small set of patients, this analysis confirmed that cumulative renal survival was significantly lower in patients harbouring the Glu/Asp + Asp/Asp genotypes [44.8 ± 1.7, 95% CI (41.5; 48.1), n = 19] compared to patients harbouring the Glu/Glu genotype [53.3 ± 2.3, 95% CI (48.8; 57.7), n = 19, P = 0.03] of the Glu298Asp polymorphism.

### Table 2. Allele frequencies and pairwise linkage disequilibrium between ENOS polymorphisms

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Allele frequencies</th>
<th>Linkage disequilibrium coefficient (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glu298Asp</td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>0.69/0.31</td>
<td>−</td>
</tr>
<tr>
<td>Intron 4 VNTR</td>
<td>0.84/0.16</td>
<td>−0.99</td>
</tr>
<tr>
<td>T-786C</td>
<td>0.61/0.39</td>
<td>0.35</td>
</tr>
</tbody>
</table>

*For each polymorphism, the frequency of the most common allele is shown first. All linkage disequilibrium coefficients are statistically significant (P < 0.001). r² is the fraction of maximum linkage that could occur between two loci, given the observed allelic frequencies (Materials and Methods).

### Table 3. Age at ESRD according to genotypes of ENOS polymorphisms in male and female ADPKD subsets (simple regression analysis)

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Genotypes</th>
<th>n</th>
<th>Age at ESRD (years)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n = 93)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu/Glu</td>
<td>40</td>
<td>53.5 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu/Asp + Asp/Asp</td>
<td>53</td>
<td>49.0 ± 1.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Intron 4 VNTR</td>
<td>bb</td>
<td>64</td>
<td>49.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ba + aa</td>
<td>29</td>
<td>53.9 ± 1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>T-786C</td>
<td>TT</td>
<td>27</td>
<td>49.6 ± 1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT + CC</td>
<td>66</td>
<td>51.5 ± 1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Females (n = 80)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu/Glu</td>
<td>41</td>
<td>51.4 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>Glu/Asp + Asp/Asp</td>
<td>39</td>
<td>53.2 ± 1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Intron 4 VNTR</td>
<td>bb</td>
<td>59</td>
<td>51.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ba + aa</td>
<td>21</td>
<td>54.2 ± 3.0</td>
<td>0.6</td>
</tr>
<tr>
<td>T-786C</td>
<td>TT</td>
<td>34</td>
<td>50.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT + CC</td>
<td>46</td>
<td>53.7 ± 1.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
Correlations between Glu298Asp genotype and clinical parameters in ADPKD

Clinical parameters related to renal function and blood pressure were obtained in subsets of ADPKD patients, in an attempt to look for clinical factors underlying the lower age at ESRD observed in patients harbouring the Glu298Asp polymorphism.

The profile of renal function loss was obtained in 22 unrelated ADPKD males (Fig. 3). As in the whole male ADPKD subset, the mean age at ESRD was significantly lower in the (Glu/Asp + Asp/Asp) subgroup (45.1 ± 1.9 years, n = 12) than in the Glu/Glu subgroup (53.1 ± 2.7 years, n = 10; P = 0.02). The 1/creatinine slope was steeper in the former subgroup (–0.0065 versus –0.0049 in the Glu/Glu subgroup).

Several parameters assessing indirectly the severity of hypertension were available in a subset of 37 ADPKD patients. As expected, a trend towards increased septum (P = 0.3) and posterior wall thickness (P = 0.1) was observed in males (n = 22) versus females (n = 15). In males, a significantly lower age at ESRD was found again in the (Glu/Asp + Asp/Asp) subgroup (46.7 ± 2.0 years, n = 12 versus 53.7 ± 2.7 years, n = 10; P = 0.04). However, no significant difference regarding indexes of left ventricular hypertrophy, estimated duration of hypertension and number of classes of anti-hypertensive drugs was observed according to genotype (data not shown).

NOS enzymatic activities and expression of eNOS in renal arteries

To substantiate the molecular mechanism underlying the influence of the Glu298Asp polymorphism, we assessed both the NOS enzymatic activity and the expression of eNOS at the mRNA and protein levels in renal arteries from ADPKD patients differing for the Glu298Asp genotype. Because the expression of eNOS is influenced by estrogens (16) and age (19), investigations were conducted on samples matched for gender and age.

A panel of well characterized monoclonal and polyclonal antibodies raised against human eNOS was used to demonstrate that eNOS is the predominant NOS isoform expressed in control and ADPKD renal arteries (Fig. 4A–C). Immunostaining in renal arteries from ADPKD patients with the Glu/Glu genotype confirmed the distribution of eNOS in the endothelium, where it co-localized with factor VIII (Fig. 4D).

<table>
<thead>
<tr>
<th>T-786C</th>
<th>Glu298Asp</th>
<th>n</th>
<th>Age at ESRD (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>Glu/Glu</td>
<td>19</td>
<td>50.4 ± 1.9</td>
</tr>
<tr>
<td>TT</td>
<td>Glu/Asp + Asp/Asp</td>
<td>8</td>
<td>47.9 ± 2.5</td>
</tr>
<tr>
<td>CT</td>
<td>Glu/Glu</td>
<td>16</td>
<td>54.2 ± 2.3</td>
</tr>
<tr>
<td>CT</td>
<td>Glu/Asp + Asp/Asp</td>
<td>37</td>
<td>48.6 ± 1.5</td>
</tr>
<tr>
<td>CC</td>
<td>Glu/Glu</td>
<td>5</td>
<td>63.0 ± 5.1</td>
</tr>
<tr>
<td>CC</td>
<td>Glu/Asp + Asp/Asp</td>
<td>8</td>
<td>52.1 ± 2.2</td>
</tr>
</tbody>
</table>

*The model including T-786C and Glu298Asp polymorphisms explains 13% of the variance of age at ESRD (r² = 0.13; P = 0.006). The effect of the Glu/Asp + Asp/Asp versus Glu/Glu genotypes across the three genotypes of T-786C is significant (P = 0.006). Values are expressed as means ± SEM.
The L-citrulline assay was used to determine Ca\textsuperscript{2+}-dependent NOS activity, which relies on eNOS and neuronal NOS (nNOS), in renal artery extracts from nine ADPKD patients matched for age (Fig. 5A). This analysis showed that Ca\textsuperscript{2+}-dependent NOS activity was reduced in ADPKD patients compared to normal controls.

**Figure 4.** Expression of endothelial NOS in renal arteries. (A) Immunoblot analyses of the expression of eNOS in extracts from BAEC (used as a positive control for eNOS) and normal human renal arteries (HRA). Two microlitres of BAEC lysate and 30 µg of HRA extract were run on 7.5% PAGE, transferred to nitrocellulose and probed either with monoclonal (left) or affinity-purified polyclonal antibodies (right) against human eNOS, or the corresponding mouse or rabbit non-immune IgG at the same dilution (mlgG and rlG, respectively). The 140 kDa band corresponding to eNOS is clearly detected with both antibodies. Non-specific bands of ∼250 kDa and <75 kDa are identified with non-immune IgG. The films were exposed for 5 min (antibodies) and 20 min (control IgG). (B) Expression of eNOS in BAEC and HRA: peptide competition analysis. Two microlitres of BAEC lysate and 30 µg of HRA extract were run on 7.5% PAGE and transferred to nitrocellulose. Identical strips were probed with the affinity-purified polyclonal antibodies against human eNOS in control conditions, or following pre-adsorption with an excess of eNOS versus an unrelated peptide. The 140 kDa band corresponding to eNOS is competed away when primary antibodies are pre-adsorbed with the eNOS peptide but not when they are pre-adsorbed with the unrelated peptide. The signal for β-actin was obtained after stripping the blots. The films for eNOS were exposed for 30 min. (C) Immunoblot analyses of nNOS, eNOS and iNOS isoforms expression in renal arteries from normal subjects and ADPKD patients. Thirty micrograms of extract obtained from renal arteries of a control subject and an ADPKD patient were run on 7.5% PAGE and probed with monoclonal antibodies against NOS isoforms. Positive controls include rat brain extract (nNOS), BAEC (eNOS) and macrophages (iNOS). In comparison with nNOS (155 kDa), eNOS (140 kDa) is the predominant isoform expressed in renal arteries. As previously reported by Combet et al. (50), the monoclonal anti-iNOS cross-reacts with an upper band corresponding to eNOS (asterisk), but iNOS (130 kDa) is not detected in this sample. (D) Co-localization of eNOS and factor VIII in the endothelium lining renal arteries from an ADPKD patient homozygous for the Glu298 allele (Glu/Glu). No specific signal is detected when the monoclonal anti-eNOS antibody is replaced with non-immune, mouse IgG. The asterisk indicates the lumen (magnification 275×).
activity was systematically decreased in renal arteries of ADPKD patients harbouring the Glu/Asp genotype (−42%, n = 3) or the Asp/Asp genotype (−63%, n = 4) as compared with two ADPKD patients harbouring the Glu/Glu genotype. Similar results were observed for total NOS activity, reflecting the minimal Ca
sup
2+-dependent NOS activity in these samples. As expected, Ca
sup
2+-dependent NOS activity decreased with age, irrespective of the genotype (data not shown).

The renal artery samples that were used for the L-citrulline assay were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to investigate the expression of eNOS by immunoblotting, using both monoclonal and polyclonal antibodies against human eNOS. As shown in Figure 5B, the Asp/Asp samples were characterized by a significant decrease in the expression of full-length eNOS at 140 kDa. This was paralleled by a major increase in the amount of an immunoreactive band for eNOS at ~70 kDa. The apparition of a similar 70 kDa band in endothelial cell lysates suggested that it might correspond to proteolytic cleavage of eNOS. It must be noted that the immunoblot pattern for eNOS in Asp/Asp samples, including the major immunoreactive band for eNOS at ~70 kDa, was identical when using the lithium dodecyl sulfate (LDS) sample buffer system (20) designed to limit acidic hydrolysis (Fig. 5B). In contrast with the modified expression of eNOS at the protein level (Fig. 5B), real-time quantitative RT–PCR revealed that ENOS mRNA expression levels were not significantly different in age-matched renal arteries belonging to the different Glu298Asp genotypes (1.08- and 1.12-fold the Glu/Glu artery level in Glu/Asp and Asp/Asp arteries, respectively) (data not shown).

The hypothesis of increased proteolytic cleavage of eNOS in renal artery samples harbouring the Asp/Asp genotype was substantiated by immunoprecipitation with polyclonal antibodies directed against the N-terminus or C-terminus domains of human eNOS, followed by immunoblotting with the monoclonal antibody against the C-terminus of eNOS (Fig. 5C). Immunoprecipitation with both antibodies yielded the full-length eNOS (140 kDa) in all samples, whereas a 70 kDa fragment containing the C-terminus of eNOS was identified in the Asp/Asp sample only. Immunoprecipitation with anti-caveolin antibodies yielded full-length eNOS in both samples, whereas no signal was observed when the precipitation was performed with non-immune rabbit IgG or in absence of primary antibody (beads only) (Fig. 5C).

The expression of eNOS was further assessed by immunostaining in renal arteries from two ADPKD males harbouring the Asp/Asp genotype (Fig. 6). In contrast with the staining observed in Glu/Glu arteries (Fig. 4D) and the preserved expression of endothelial factor VIII (Fig. 6A, C and E), endothelial staining for eNOS was reduced in renal arteries (B) and not detected in intra-parietal vasa vasorum (D and F).

**DISCUSSION**

We have investigated the effect of the three most characterized polymorphisms of ENOS on the age at ESRD in a large series of unrelated ADPKD patients. No effect on the age at ESRD was found for the intron 4 and T-786C polymorphisms. In contrast, the Glu298Asp polymorphism was associated with a significantly lower age at ESRD in ADPKD males and was over-represented in the subset of ADPKD males reaching ESRD before age 50 years. The effect of the Glu298Asp polymorphism in males with ADPKD was confirmed in subgroups of unrelated patients linked to PKD1, as well as by a renal survival analysis in PKD1-linked families.

The Glu298Asp polymorphism of ENOS has already been associated with hypertension (21), myocardial infarction (22) and carotid atherosclerosis (23), three conditions characterized by endothelial dysfunction. Our data support an influence of the Glu298Asp polymorphism on the progression of ADPKD, another disease associated with endothelial dysfunction (13). The production of NO through eNOS is regulated by estrogen (24,25), which might explain a gender sensitivity to NO (16) and our observation that the effect of the Glu298Asp polymorphism was restricted to ADPKD males. These data are also consistent with the observation that, in the Han:SPRD rat model of ADPKD (26), NOS inhibition with L-NAME had a deleterious effect on the progression of renal disease only in males (27).

In order to find a molecular basis for the lower age at ESRD observed in patients harbouring the Glu298Asp
polymorphism, we investigated activity and expression of eNOS in renal arteries of ADPKD males according to the genotype at the Glu298Asp locus (Fig. 5). Contrasting with a similar expression of $ENOS$ at the mRNA level, the Ca$^{2+}$-dependent NOS activity was systematically lower in patients with the Glu/Asp or Asp/Asp genotype. Furthermore, the Asp/Asp samples were characterized on immunoblot by a decrease in full-length eNOS and a parallel increase in a smaller immunoreactive band at 70 kDa, which was identified as a proteolytic fragment of eNOS by immunoprecipitation. The modified expression of eNOS in these samples was confirmed by immunostaining (Fig. 6), with a reduced endothelial staining in renal arteries and a lack of signal in the endothelium lining vasa vasorum.

These data confirm the in vitro demonstration of increased degradation of eNOS in patients harbouring the Asp298 polymorphism (28). The mechanism of this degradation remains to be elucidated. The glutamic acid at position 298 of eNOS is conserved among species. Recent structural data suggest that the residue at 298 is located between the $\beta$-strands 8 and 9 of eNOS, and is thus fully solvent-exposed, which could make it more sensitive to proteolysis (29). Alternatively, the Asp298 residue could increase the sensitivity of eNOS to acidic hydrolysis as it leads to the formation of an Asp298–Pro299 motif, known to sensitize proteins to acidic hydrolysis (20). However, the latter hypothesis appears unlikely, as our immunoblot results were similar when using the LDS-electrophoresis sample buffer to maintain a neutral pH throughout the experiment (Fig. 5). Taken together, these results suggest that lower enzymatic activity and/or partial cleavage of eNOS could be responsible for a decreased NO release and increased endothelial dysfunction in patients harbouring the Asp298 allele. In the absence of clinical arguments suggesting increased systemic blood pressure, one might postulate that the accelerated renal function degradation observed in these patients could be mediated by a local effect on renal microcirculation.

The two other polymorphisms of $ENOS$ tested in this study, intron 4 VNTR and T-786C, do not appear to influence the renal phenotype of ADPKD. The finding of an increased age at ESRD in ADPKD males harbouring the a allele of intron 4 VNTR, previously associated with hypertension (30) and

![Figure 6](image-url)
coronary artery disease (31), was not confirmed by multiple regression analysis. It probably reflects linkage disequilibrium with the Glu298 allele (Table 2). We found no effect of the T-786C promoter polymorphism, which has been associated with a modest (40%) decrease in promoter activity and decreased plasma NO metabolites in some studies (32) but not in others (33). It must be noted that the mouse equivalent of ENOS is located on chromosome 5, thus outside the modifier loci identified in murine models of polycystic kidney disease (9,10). However, these models are all recessive and thus are not a faithful equivalent of human ADPKD.

Only a few candidate genes likely to influence the progression of human ADPKD have been investigated thus far (34). In vitro data have suggested that CFTR channel mediates fluid secretion in ADPKD cysts (35) and an apparent protective effect of rare CF mutations on renal decline in ADPKD has been reported (36). However, we were unable to confirm that the most common ΔF508 mutation of CF affects the progression of ADPKD (37). Similarly, initial results suggesting an effect of the ACE I/D polymorphism in ADPKD (38) have been disputed and now appear unlikely (39,40). Such conflicting results might be, at least in part, explained by the lack of power due to the small number of patients included and/or the inclusion of patients belonging to a small number of large PKD1 families (34). This last strategy could indeed lead to spurious results due to under- or over-representation of a given polymorphism in a few large families (41).

In order to overcome these potential biases, we primarily included a large number of unrelated patients, even from uninformative families in terms of linkage to PKD1 or PKD2. Nevertheless, linkage analysis was performed in all informative families, in order to exclude PKD2 families. The latter accounted for 15% of the families tested, in agreement with the expected prevalence in our population (6). Whereas an over-representation of PKD2 patients in the Glu/Glu group subset would still contribute to a higher age at ESRD, this hypothesis appears unlikely, as the mean age of the Glu/Glu group (53.5 years) is similar to that found in PKD1-linked patients (6). Furthermore, the effect of the Glu298Asp polymorphism is maintained when examining subsets of ADPKD males linked to PKD1 and those reaching ESRD before the age of 45 years. Previous studies in a similar population have indeed shown that the prevalence of PKD2 mutations is negligible in the latter (6,34,38). Finally, a Kaplan–Meier analysis performed on all affected males belonging to our PKD1-linked families confirmed that cumulative renal survival was significantly lower in patients harbouring the Asp allele of the Glu298Asp polymorphism.

It must be emphasized that the association of the Glu298Asp polymorphism with a faster renal decline in ADPKD males cannot be extrapolated without further studies in other populations with different genetic backgrounds. This is particularly true if the Glu298Asp polymorphism is not the causal polymorphism, but only a marker in linkage disequilibrium with the modifier locus. In particular, it would be worthwhile to look for the influence of ENOS in ADPKD patients from Japan, in which the Asp298 allele is significantly less frequent than in Caucasians (allelic frequency: 14 versus 31%, P < 0.001) and is not in linkage disequilibrium with the intron 4 VNTR (A.Persu and S.Horie, unpublished data).

In conclusion, in male patients from a large ADPKD subset from Belgium and the north of France, the Glu298Asp polymorphism of ENOS is associated with a 5 year lower mean age at ESRD. The NOS activity is significantly decreased in renal artery samples from ADPKD males harbouring the Asp298 allele, in association with post-translational modifications and a partial cleavage of eNOS. It is tempting to speculate that the resulting decrease in NO production may enhance the endothelial dysfunction associated with ADPKD, leading to alteration of intrarenal and/or systemic haemodynamics. This in turn will result in a faster decline in renal function. If the effect of the Glu298Asp polymorphism is confirmed in independent populations, ENOS would appear as the first modifier gene in ADPKD.

MATERIALS AND METHODS

Study population

Patients were recruited from September 1998 to September 2000 in Saint-Luc Academic Hospital, Brussels (Belgium), U.Z. Gasthuisberg, Leuven (Belgium) and Necker Hospital, Paris (France). All Caucasian patients affected with ADPKD on renal replacement therapy (dialysis or renal transplantation) in the three centres during the recruitment period were included. The diagnosis of ADPKD was established on the basis of bilateral enlarged cystic kidneys and a family history suggestive of autosomal dominant inheritance (1). The age at ESRD was defined as the age at starting renal replacement therapy (creatinine clearance 10 ml/min). All patients recruited in this first phase were unrelated. A detailed follow-up for at least 2 years before ESRD was available for all patients included.

Linkage analysis for PKD1 and PKD2 was performed in informative families, whenever possible, using microsatellites SM7, AC2.5 and KG8 for PKD1 (42) and D4S1534, D4S231, D4S414, and D4S1563 for PKD2 (43). LOD scores were calculated by the Mlink program (Columbia University, New York, NY). Data available from the linked markers were combined by means of a Bayesian weighting formula to estimate the likelihood that a family shows linkage to PKD1 or PKD2. The prevalence estimates used for PKD1, PKD2 and a hypothetical PKD3 gene were 0.84, 0.15 and 0.01, respectively (5,6). In order to perform a Kaplan–Meier renal survival analysis, we subsequently recruited all affected males (according to the diagnostic criteria for ADPKD defined above) within the families linked to PKD1.

The use of DNA and renal artery samples was approved by the Ethical Review Board of each centre involved, and informed consent was obtained from all patients included.

DNA extraction and genotyping for ENOS polymorphisms

DNA was extracted from peripheral blood samples (Gentra, Minneapolis, MN). PCR for ENOS polymorphisms was carried out in a 20 µl volume with 100 ng of genomic DNA, 10 µM of each primer, 1.25 mM dNTP (Roche Diagnostics, Mannheim, Germany), 1 U Taq polymerase (Roche) and 2 µl of 10x buffer containing 15 mM MgCl2 (Roche). Genotyping for the intron 4 VNTR was performed using primers and conditions described previously (31,44). Genotyping for Glu298Asp was
performed by PCR, followed by BanII (Life Technologies, Carlsbad, CA) digestion, and additional control using MboI digestion, as described (21). Genotyping for T-786C was obtained by PCR followed by allele-specific oligonucleotide hybridization (ASO), as described earlier (45). Three patients found to harbour the three possible genotypes (TT, CT and CC) by sequencing were used as positive controls. The localization of the three polymorphisms in ENOS and representative gels or ASO are shown in Figure 1.

**Statistical analysis**

All data were analysed using the SPSS statistical software (version 10.0; SPSS, Chicago, IL), and P-values ≤0.05 were considered as significant. The ages at ESRD were normally distributed in males and females, and compared by two-tailed Student’s t-test. For each polymorphism, allele frequencies were calculated from the genotype. Allele and genotype distribution in male and female subsets were compared by chi-square ($\chi^2$) test. As functional changes induced at the protein/mRNA level are observed whether the polymorphisms were present at the homozygous or the heterozygous state (28,32,46), statistical analysis was performed by combining heterozygotes and homozygotes rare allele carriers.

Hardy–Weinberg equilibrium was tested by a $\chi^2$ test. Pairwise linkage disequilibrium between the different polymorphisms was tested and the linkage coefficient $D'$ was obtained as described. $D'$ is the fraction of maximum linkage that could occur between two loci, given the allelic frequencies. The sign added in front of $D'$ is positive if the less frequent alleles at both loci are preferentially associated and negative if the less frequent allele at one locus is associated with the most frequent allele at the other locus (47).

Simple linear regression analysis was used to assess the individual effect of each ENOS polymorphism (independent variable) on age at ESRD (dependent variable). Multiple linear regression analysis was performed to investigate the joint effects of ENOS polymorphisms on the age at ESRD; variables that significantly influenced the age at ESRD were selected by a forward stepwise procedure. The increase in the model $r^2$ was used to explain the proportion of the variance added by each polymorphism, and allowed us to select the model which explains the highest proportion of the variance. To deal with the categorical nature of a polymorphism, dummy variables were generated for each polymorphism.

Haplotype analysis was performed by analysis of variance for the Glu298Asp and T-786C polymorphisms (48). In the absence of the parental genotypes, four haplotypes (H1–H4) were constructed as follows: H1 (Glu in T background) = Glu/Glu–TT genotype; H2 (Asp in T background) = Glu/Asp–TT and Asp/Asp–TT genotypes; H3 (Glu in C background) = Glu/Glu–CT and Glu/Glu–CC genotypes; and H4 (Asp in C background) = Glu/Asp–CT, Asp/Asp–CT, Glu/Asp–CC and Asp/Asp–CC genotypes. The H1 haplotype (genotype Glu/Glu–TT) was used as the reference haplotype (48).

Differences in genotype distribution between patients having reached ESRD before or after 50 years of age were tested by the $\chi^2$ test. Cumulative renal survival analysis in males belonging to PKD1-linked families was performed using the Kaplan–Meier method, and the log-rank test was used to compare renal survival according to the genotype at the Glu298Asp locus. The influence of the genotype on echocardiographic parameters (either normally distributed, or becoming normal after log-transformation) was tested using two-tailed Student’s t-test. Continuous variables were expressed as mean ± SEM.

**Clinical parameters**

The rate of decline in renal function loss over time was indicated by the 1/plasma creatinine slope for individual patients. Patients were included in the analysis only if at least five determinations of plasma creatinine were available before ESRD, and the 1/plasma creatinine slope was obtained by linear regression. Septum and posterior wall thickness, as well as telesystolic and telediastolic diameters, were measured by M-Mode echocardiography (49), performed at the time of initiation of dialysis in ADPKD patients (Acuson Sequoia, Moutain View, CA). Classes of antihypertensive therapy included diuretics, $\beta$-blockers, calcium antagonists, ACE inhibitors and central acting agents.

**Cell and tissue samples and processing**

Renal artery samples from nephrectomy specimens were obtained in 12 ADPKD males at the time of transplantation, and in four controls (two males, two females) at the time of surgery for renal cell carcinoma. Part of each sample was fixed for 6 h at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, rinsed, and embedded in paraffin (37). Most of the sample was used for protein extraction (for NOS assay and immunoblot), as previously described by Combet et al. (50). Lysates from bovine aortic endothelial cells (BAEC) were obtained from Transduction Laboratories (Lexington, KY). The protein concentrations were determined using the Bradford method (Bio-Rad, Melville, NY) with BSA as standard.

**Antibodies**

NOS isoforms were detected with mouse monoclonal antibodies against human eNOS (C-terminus) and nNOS, and mouse inducible NOS (iNOS) (Transduction Laboratories) and affinity-purified rabbit polyclonal antibodies against the N-terminus (Santa Cruz Biotechnology, Santa Cruz, CA) or C-terminus (Transduction Laboratories) of human eNOS (50). Both the monoclonal (C-terminus) and polyclonal (N-terminus) antibodies were used for detecting eNOS by immunoblotting. Additional antibodies included rabbit antibodies against human Factor VIII (Dako, Glostrup, Denmark); monoclonal anti-$\beta$-actin (Sigma, St Louis, MO) and rabbit polyclonal anti-human caveolin 1 (Transduction Laboratories). Peroxidase-labeled IgG were from Dako and avidin–biotin peroxidase kits from Vector (Burlingame, CA).

**Immunohistochemistry**

The reactivity for eNOS and Factor VIII in renal arteries from ADPKD patients was investigated using immunoperoxidase, as described by Persu et al. (37). Following incubation in 0.3% H$_2$O$_2$ (30 min) and 10% normal serum in PBS (20 min) at room temperature, dehydrated paraffin sections were incubated successively with primary antibodies, biotinylated goat anti-rabbit or horse anti-mouse IgG, avidin–biotin peroxidase complex, and after washing, aminoethylcarbazole (Vector). Sections were examined under a Leica DMR coupled to a
Leica MPS60 photomicrographic system (Leica, Heerbrugg, Switzerland). The specificity of the immunolabeling was confirmed by incubation without primary or secondary antibody, or with non-immune rabbit or mouse IgG (Vector).

**Measurement of NOS activity**

NOS activity was assayed in renal artery samples by the conversion of L-[^3H]arginine (Amersham, Little Chalfont, UK) to L[^3H]citrulline as previously described by Combet et al. (50). The NOS activity (pmol citrulline produced/mg protein/min) was calculated from the counts obtained with and without 1 mM L-NMMA. Assays were performed with 1 mM CaCl₂ and, alternatively, without Ca²⁺ (0 mM CaCl₂, 2 mM EDTA, 2 mM EDTA) to measure total versus Ca²⁺-independent NOS activities and calculate Ca²⁺-dependent NOS activity. Both inter-assay and intra-assay variability were <10%. All determinations were performed in duplicate in the samples obtained from nine ADPKD patients.

**Immunoblotting**

SDS–PAGE and immunoblotting were performed as described previously (20,50), using either the Laemmli loading buffer or the LDS sample buffer. Cell lysates and renal artery samples prepared as described above were separated by SDS–PAGE and transferred to nitrocellulose. The membranes were blocked for 30 min at room temperature, and incubated overnight at 4°C with the primary antibody. After washing, membranes were incubated for 1 h at room temperature with peroxidase-labeled secondary antibodies (1:5000 dilution), and immunoblots were visualized with enhanced chemiluminescence (Amersham). The specificity of the bands corresponding to eNOS was verified by incubation (i) with non-immune rabbit or mouse IgG (Vector) and (ii) with affinity-purified anti-eNOS antibodies pre-adsorbed with a 5-fold excess of the gene, expressed as fold-variation over control, was calculated above a predefined threshold). Relative quantitation for a given gene, expressed as fold-variation over control, was calculated using the 2⁻ΔΔCt formula after normalization to GAPDH (ΔCt) and determination of the difference in Ct (ΔΔCt) between control and polymorphism-bearing arteries.

**ACKNOWLEDGEMENTS**

We are indebted to the ADPKD patients, their families, and the nurses and staff of the nephrology units involved. We would like to thank Professors X.Jeunemaitre, J.-P.Grünfeld, M.Vikkula and M.Jadoul for fruitful discussions, and F.Meunier for statistical advice. We are grateful to Drs J.Bacon, J.-L.Christophe, Ch.Cuvelier, J.-J.Lafontaine, G.Loute, F.Regisster, J.Vanwellegem and the members of the Leuvense Samenwerking Groep voor Transplantatie for information and DNA samples. Professor J.-P.Squifflet, and H.Debraix, L.Wenderickx and A.Herelixka for excellent technical like to thank Professors X.Jeunemaitre, J.-P.Grünfeld, M.Vikkula and M.Jadoul for fruitful discussions, and F.Meunier for statistical advice. We are grateful to Drs J.Bacon, J.-L.Christophe, Ch.Cuvelier, J.-J.Lafontaine, G.Loute, F.Regisster, J.Vanwellegem and the members of the Leuvense Samenwerking Groep voor Transplantatie for information and DNA samples. Professor J.-P.Squifflet, and H.Debraix, L.Wenderickx and A.Herelixka for excellent technical assistance. R.W. is a Research Associate from the FNRS. This work was supported by the Communauté Française de Belgique, the Société de Néphrologie, the Belgian agencies FNRS and FRSM, the Action de Recherches Concertées 00/05-260, the Xunta de Galicia (XUGA 90201B98) and Ministerio de Ciencia y Tecnologia (PM98-0028).

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