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# Allelic genes involved in artery compliance and susceptibility to sporadic abdominal aortic aneurysm

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**Abstract**

Vascular smooth muscle cells (VSMCs) synthesize elastin (ELN), major protein of aortic tunica media which confers strength and elasticity to aortic wall. Protein loss or distortion is typical in aneurysm tunica media. Transforming growth factor β1 (TGFβ1) inhibits growth and connective protein expression of abdominal VSMCs cultures. Also, in atherogenic studies, estrogen (but not estrogen plus progestin) treatments inhibit aortic collagen accumulation and elastic loss, risk factors to subsequent aortic enlargement. Therefore, polymorphisms of ELN, estrogen receptor α (ERα) and β (ERβ), progesterone receptor (PR) and TGFβ1 genes and their products may be involved in the abdominal aortic aneurysm (AAA) development. Using PCR-RFLP method, we analyzed ELN *Rma*I (exon 16), ERα *Pvu*II-*Xba*I (intron 1), ERβ *Alu*I (exon 8), PR *Taq*I (intron 7) and TGFβ1 *Bsu*36I (−509 bp, promoter) polymorphisms in 324 Caucasian male subjects: 225 healthy controls (mean age 71.20 ± 6.85 years) and 99 unrelated AAA patients (mean age 69.8 ± 7.1 years). No difference in ELN, ERα, PR and TGFβ1 allele frequencies was observed in AAA patients versus controls ($P > 0.05$). However, because possessing at least an ERβ *Alu*I restriction site was statistically associated to AAA onset ($\chi^2 = 5.220$; OR = 1.82, $P < 0.05$), ERβ polymorphism was proposed as genetic determinant in the AAA susceptibility.


*Keywords:* Aneurysm; Artery; Elastin; Estrogen receptor; Polymorphism; Progesterone receptor; Transforming growth factor beta

## 1. Introduction

The arterial aneurysm is localized abnormal dilation of blood vessel that most frequently occurs in the abdominal aorta. The abdominal aorta aneurysm (AAA) is progressive degenerative disorder primarily affecting aortic tunica media with structural wall distortion, collagen accumulation and elastic layer loss. Given these tunica media wall changes, aorta is less elastic and compliant which is major risk factor of subsequent aortic enlargement.

The vascular smooth muscle cell (VSMC), the most abundant cell type in the aortic tunica media, is primarily involved in arterial metabolism and thus, in aneurysm wall degeneration. In fact, VSMCs synthesize extracellular matrix proteins such as elastin (ELN) and collagen which confer strength and elasticity to aortic wall [1–3].

In vertebrates, tunica media VSMCs arise from two embryological origins, the cardiac neural crest and the mesoderm [4]. VSMCs that differentiate from cardiac neural crest are referred to as ectodermal (Ect) VSMCs, while those that originate from mesoderm are called mesenchymal (Mes) VSMCs. The two VSMC types are not equally distributed within the large elastic outflow arteries. Aorta and its arteries are made up of a mixture of Ect and Mes VSMCs, with Ect prevailing in the proximal (thoracic) segments and Mes prevailing in the abdominal aorta and iliac arteries [5].

Beyond their rigid aortic localization, the two VSMC lineages differ both in structural protein synthesis (i.e. collagen and ELN) and in proliferative responses to growth

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factors [1,6]. For example, while transforming growth factor β1 (TGFβ1 increases mitosis markers in Ect VSMCs, it does not produce growth stimulation but rather growth inhibition in Mes VSMCs [6].

Physical aortic properties vary as heart distance function highly depending on interstitial collagen and ELN content. Thoracic aorta without age-atherosclerosis damage is an ELN-rich organ while abdominal aorta is largely collagenous [1]. This aortic wall structure is due to specific VSMC-lineage synthesis of structural proteins. ELN synthesis by VSMCs decreases 10-fold from thoracic to abdominal aorta, while collagen production shows opposite trends increasing along the aorta axis [1]. Possibly, the higher incidence in aneurysms at the abdominal aorta level may depend to lower aortic compliance compared to the thoracic segment.

Given the central role of ELN and TGFβ1 proteins in VSMC biology and then, in aortic wall trophism, genes encoding these two proteins become easy candidates for risk of aneurysm disorders.

The etiology of aortic aneurysm is largely unknown, but a certain influence of genetic factors has been established. For examples, there are families exhibiting autosomal dominant or recessive inheritance pattern of the disorder and for most of families, AAA incidences are similar in both sexes [7]. On the other hand, the most common sporadic AAA is much frequent in males than in females. Before 65 years of age, AAA is almost male prerogative while then, AAA incidence is progressively increased in female sex too. Because AAA incidence in female population over 65 years old is about 6% (versus 14% for male), AAA onset in women appears to lag 10 years behind that of men [7]. This AAA onset trend is possibly due to protective effects of female sex steroids (i.e. estrogens and/or progestins) over their lifetime [7].

Based on the potential protective role of female sex hormones for AAA development, together with the fact that most steroid bioeffects are mediated by their nuclear receptors (i.e. estrogen receptor α (ERα), β (ERβ) and progesterone receptor (PR)), we hypothesized ERα, ERβ and PR functions might contribute to disease. In this view, we examined possible associations between AAA development and allelic variants of genes involved in aortic VSMCs physiology such as ELN, ERα, ERβ, progesterone receptor (PR), and TGFβ1 genes [8–10].

## 2. Materials and methods

### 2.1. Subjects

The genotype analysis was carried out in 324 Caucasian subjects: 225 healthy controls (mean age 71.20 ± 6.85 years) and 99 patients (mean age 69.8 ± 7.1 years) affected by AAA and surgically treated in the University Hospital of Pisa between 1999 and 2001. All 324 subjects analyzed were unrelated Italian males living in the Tuscany region. After approval from the local Institutional Review Board, informed consent was obtained from all subjects prior to the study. Before undergoing to surgical intervention, clinical AAA diagnosis was confirmed by ecographic and spiral computed tomographic (CT) scan evaluations. Antero-posterior and lateral aortic diameter values (mean ± S.D.) were increased to 50.78 ± 11.66 mm and 50.54 ± 13.35 mm, respectively, than control standard (18.0 ± 5.0 mm). From all studies subjects, a blood-EDTA sample was performed and stored at −20 °C until genetic analysis.

### 2.2. Genotyping

Constitutive lymphocyte DNA was extracted from blood samples by standard phenol/chloroform procedure and *Rma*I, *Pvu*II, *Xba*I, *Alu*I, *Taq*I and *Bsu*36I genotypes (of ELN, ERα, ERβ, PR and TGFβ1 genes, respectively) were determined by PCR methods [11–13]. Specific details of restriction fragment length polymorphism (RFLP) techniques are following reported.

Regard ELN *Rma*I polymorphism, we used oligonucleotide primers (forward, 5′-TACTTGGCTCCCTTCCCT-3′; reverse, 5′-CTCACGGGAAATGCCAAC-3′) for each reaction at 0.4 μM. PCR was performed from 0.1 μg of DNA in 50 μl of buffer solution (10 mM Tris–HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1% Triton X-100, and 200 μM dNTPs), 1 U of *Taq* polymerase (Promega, Madison, WI) through 35 cycles by the following steps: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 90 s. PCR products, 194 bp fragments, were digested with 20 U of *Rma*I endonuclease enzyme (Promega, Madison, WI), and then, detected by denaturing polyacrylamide gel.

To analyze ERα gene polymorphisms, 1 U of *Taq* polymerase and 0.4 μM of oligonucleotide primers (forward, 5′-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3′; reverse, 5′-TCTTTCTCTGCCACCCTGGCGTCGATTATCT-GA-3′) were used for each PCR reaction. 0.1 μg of genomic DNA were PCR-amplified in 50 μl of buffer solution (10 mM Tris–HCl, pH 9, 50 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100, and 200 μM each of the four deoxyribonucleotides (dNTPs)). PCR reaction was performed through 30 cycles by the following steps: denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 90 s. The PCR product was a 1.3 kbp inton 1-exon 2 fragment of ERα gene. After amplification PCR products were digested with 10 U of either *Pvu*II or *Xba*I restriction endonucleases (Roche Molecular Biochemicals, I) and electrophoresed in a 2.0% agarose gel.

The 1730(A-G) *Alu*I RFLP of ERβ 3′ untranslated region (3′ UTR) were analyzed using oligonucleotide primers (forward, 5′-CAAGTCCATCACGGGGT-3′; reverse, 5′-AGATGAACCCAGGCTGGTG-3′) for each reaction at 0.4 μM. PCR was performed from 0.1 μg of DNA in 50 μl of buffer solution (10 mM Tris–HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1% Triton X-100, and 200 μM dNTPs), 1 U of *Taq* polymerase through 35 cycles by the following steps: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 90 s. PCR products were 168 bp fragments.

Then, they were digested with 20 U of *Alu*I endonuclease enzyme (Promega, Madison, WI), and detected by 3.5% agarose gel electrophoresis.

The PR *Taq*I RFLP analysis (*PROGINS* allele) was based on PCR-amplification of a fragment encompassing the 306 bp *Alu* insertion polymorphism in intron 7 of PR gene (forward primer, 5′-GCCTCTAAAATGAAAGGCAGAAAGC-3′; reverse primer, 5′-GCGCGTATTTTCTTGCTAAATGTCTG-3′). All PCR products were carried out in 25 μl aliquots containing about 0.1 μg of constitutive DNA, reaction buffer (10 mM Tris–HCl pH 9, 50 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100), 200 μM dNTPs and 1 U of *Taq* polymerase. The amplification was for 30 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. After amplification PCR products were digested with 10 U of *Taq*I restriction endonuclease (Roche Molecular Biochemicals, I) and electrophoresed in 3.5% agarose gel.

The TGFβ1 *Bsu*36I polymorphism was PCR-amplified using forward 5′-CAGACTCTAGAGACTGTCAG-3′ and reverse 5′-GTCACCAGAGAAAGAGGAC-3′ primers. Aliquots of 1.0 μg of genomic DNA were added to 20 μl reaction consisting of 1X *Taq* polymerase buffer, 0.5 U *Taq* polymerase, 50 nmol each dNTP and 6.25 pmol each above PCR primers. Then, samples were amplified for 35 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, followed by an extension period of 10 min at 72 °C. PCR products were digested with 20 U of *Bsu*36I endonuclease enzyme (Promega, Madison, WI) and detected by 3.5% agarose gel electrophoresis.

The presence of the restriction site for each endonuclease was conventionally indicated with lowercase letter (*r*, *p*, *x*, *a*, *t* or *b*, respectively for *Rma*I, *Pvu*II, *Xba*I, *Alu*I, *Taq*I, and *Bsu*36I restriction endonucleases), while uppercase letter (*R*, *P*, *X*, *A*, *T* or *B*) indicated the absence of the restriction site. Subjects therefore, were scored as *rr*, *pp*, *xx*, *aa*, *tt* and *bb* homozygotes, *Rr*, *Pp*, *Xx*, *Aa*, *Tt* and *Bb* heterozygotes, *RR*, *PP*, *XX*, *AA*, *TT* and *BB* homozygotes according to their specific digestion pattern.

### 2.3. Statistical analysis

Statistical analysis was performed using cross-tabulation, odds ratio (OR) and standard chi-square ($\chi^2$) tests. Differences in means were considered statistically significant for $P$ values < 0.05. All statistical analyses were performed by using Statistica 5.1 software (Statsoft Inc., Tulsa, OK).

## 3. Results

In our Italian population, no significant differences were observed between AAA patients and healthy sex-matched controls with respect to age, height, weight, systolic/diastolic blood pressure, heart rate, glycemia and LDL/HDL cholesterol levels (data not shown).

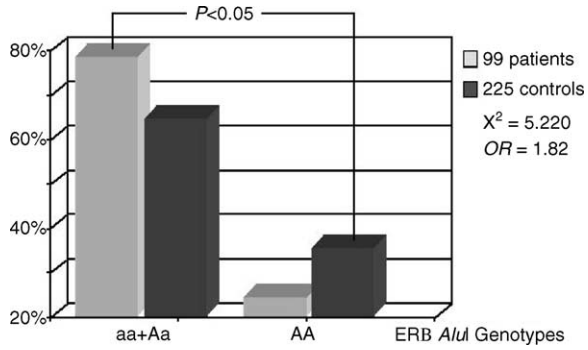


Fig. 1. *Alu*I genotype distribution of ERβ 3′ UTR polymorphism. Possessing at least a ERβ *Alu*I restriction site (*Alu*I *a* allele) was significantly associated to AAA susceptibility ($\chi^2 = 5.220$, OR = 1.82, $P < 0.05$).

Polymorphisms of ELN, ERα, ERβ, PR and TGFβ1 genes were analyzed for *Rma*I, *Pvu*II-*Xba*I, *Alu*I, *Taq*I and *Bsu*36I RFLPs respectively, exhibiting genotype distributions similar to what reported in Caucasian populations [11–13]. No-significant departures from Hardy–Weinberg equilibrium were observed. ELN, ERα, PR and TGFβ1 gene polymorphisms showed allele frequency and genotype distribution not statistically different between AAA patients and healthy controls ($P > 0.05$) (Table 1).

On the contrary, distribution of ERβ *Alu*I polymorphism differed in AAA patients and healthy controls. As reported in Table 1, ERβ *Alu*I allele frequencies for AAA patients were *A* 51% and *a* 49% whereas for controls they were *A* 59.1% and *a* 40.9% ($P < 0.05$). Regarding ERβ *Alu*I genotype distribution, *AA*, *Aa* and *aa* subjects were 22.2%, 57.6% and 20.2% in AAA group while 33.8%, 50.1% and 16.1% in control group, respectively ($P < 0.05$). As showed in Fig. 1, a higher AAA incidence was observed in subjects carrying the at least an ERβ *Alu*I restriction site (ERβ *Aa* and *aa* genotypes) compared with subjects with ERβ *AA* genotype ($\chi^2 = 5.220$; OR = 1.82, $P < 0.05$), assuming dominant effect of *Alu*I *a* allele.

No differences were observed between ELN, ERs, PR and TGFβ1 genotypes and clinical data (i.e. age, height, weight, systolic/diastolic blood pressure, heart rate, glycemia and LDL/HDL cholesterol levels) in AAA patient and control group (data not shown).

## 4. Discussion

To our knowledge, this is the first study investigating ELN, TGFβ1, ERs and PR gene polymorphisms in the sporadic AAA onset. While no association was found between ELN, TGFβ1, ERα and PR polymorphisms and AAA onset, *aa* and *Aa* genotypes of ERβ *Alu*I polymorphism were more frequent in patients than healthy sex-matched controls. Our data hence suggest that having at least an *a* allele increases the risk 1.82 times for AAA development supporting the notion that estrogens may influence arterial metabolism.

Table 1
Allele frequencies and genotype distributions of ELN, ERα, ERβ, PR and TGFβ1 genes in 99 unrelated patients affected by AAA compared to 225 healthy sex and age-matched controls

| Gene polymorphism | Genotype distribution | | | Allele frequency | |
|---|---|---|---|---|---|
| ELN *Ram* I RFLPs | *RR* (Gly/Gly) | *Rr* (Gly/Ser) | *rr* (Ser/Ser) | *R* allele | *r* allele |
| AAA patients | 37 (37.4%) | 44 (44.4%) | 18 (182%) | 0.596 | 0.404 |
| Male controls | 79 (35.1%) | 109 (48.4%) | 37 (16.5%) | 0.593 | 0.407 |
| ERα *Pvu*II RFLPs | *PP* | *Pp* | *pp* | *P* allele | *p* allele |
| AAA patients | 20 (20.2%) | 44 (44.4%) | 35 (35.4%) | 0.424 | 0.575 |
| Male controls | 48 (21.3%) | 111 (49.3%) | 66 (29.4%) | 0.460 | 0.540 |
| ERα *Xba*I RFLPs | *XX* | *Xx* | *xx* | *X* allele | *x* allele |
| AAA patients | 16 (16.2%) | 41 (41.4%) | 42 (42.4%) | 0.369 | 0.631 |
| Male controls | 46 (20.4%) | 101 (44.9%) | 78 (34.7%) | 0.429 | 0.571 |
| ERβ *Alu*I RFLPs | *AA* | *Aa* | *aa* | *A* allele | *a* allele |
| AAA patients | 22 (22.2%) | 57 (57.6%) | 20 (20.2%) | 0.510 | 0.490 |
| Male controls | 77 (33.8%) | 112 (50.1%) | 36 (16.1%) | 0.591 | 0.409 |
| PR *Taq*I RFLPs | *TT* | *Tt* | *tt* | *T* allele | *t* allele |
| AAA patients | 2 (2.0%) | 29 (29.3%) | 68 (68.7%) | 0.167 | 0.833 |
| Male controls | 6 (2.7%) | 59 (26.2%) | 160 (71.1%) | 0.158 | 0.842 |
| EGFβI *Bsu*36I RFLPs | *BB* | *Bb* | *bb* | *B* allele | *b* allele |
| AAA patients | 14 (14.1%) | 49 (49.5%) | 36 (36.4%) | 0.611 | 0389 |
| Male controls | 42 (18.7%) | 125 (55.5%) | 58 (25.8%) | 0.535 | 0465 |

Aneurysm changes in the aortic wall are especially apparent in the load-bearing media in which there is wide disorganization with loss of orderly arrangement of elastic fibers. ELN protein is major determinant of arterial distensibility forming the principal component of media elastic fibers [14]. Interestingly, Hanon et al. reported significant decrease in carotid artery distensibility in subjects with at least one *Rma*I *R* allele ($Ser^{422}$) of ELN gene [12]. In addiction, because ELN gene expression is modulated by TGFβ1 protein [15–16] and TGFβ1 circulating concentration is dependent to *Bsu*36I polymorphism at −509 bp of TGFβ1 promoter [11], we supposed that functional allelic variants of ELN and TGFβ1 genes could possibly segregate with different AAA risk. However, our findings do not support this hypothesis and in our opinion, no more studies are needed to address it.

Beneficial effect of estrogens and progestins on the aortic trophism such as other major effects on vascular system are well known and mainly mediated by their nuclear receptors (i.e. ERs and PR) [9–10]. Knockout studies demonstrated a major role of ERβ and PR genes in vascular physiology [17–18]. In primates fed with atherogenic diets, estrogen treatment inhibited detrimental connective changes associated with atherosclerosis regression (i.e. collagen accumulation and ELN loss) in the abdominal aorta, whereas progestin addition to estrogen regimen completely abolished the estrogen's atheroprotective effects [19]. Given these changes, less elastic or compliant aorta could determine subsequent aortic enlargement, representing a risk factor for AAA development.

Since the discovery that allelic variants of genes encoding for steroid receptors (i.e. ERs and PR) are associated with expression of sex steroid-responsive systems, the polymorphisms of these genes have been postulated as candidate risk markers for developing a number of chronic steroid-responsive disorders.

The human ERα gene is located on chromosome 6p25.1, comprises eight exons, and spans more than 140 kb [20]. Two single nucleotide polymorphisms have been identified in intron 1 of ERα gene: a T-397C polymorphism that is recognized by restriction *Pvu*II endonuclease [T and C alleles correspond to the presence (*p* allele) and the absence (*P* allele) of *Pvu*II restriction site, respectively] and an A-351G polymorphism that is recognized by *Xba*I [A and G alleles correspond to the presence (*x* allele) and the absence (*X* allele) of *Xba*I restriction site, respectively].

The functional significance of *Pvu*II-*Xba*I polymorphisms is not clear. *Pvu*II *P* allele disrupts a potential recognition site for transcriptional factor AP4 which recognizes CAGCTG sequence. On this regard, two studies recently detected enhancer activities in intron 1 of ERα gene. These enhancer activities differed among *Pvu*II-*Xba*I RFLPs, *P* allele confers higher transcriptional activity than *p* allele in gene reporter constructs [21–22]. In addiction, in some but not all studies, ERα *Pvu*II-*Xba*I RFLPs, alone or in combination, have been associated with bone mass in post-menopausal women or pre-menopausal women which is a clinical parameter estrogen-sensible [20].

Although the present study did not find any association between ERα intron 1 alleles and AAA developmental risk, we suggested that ERβ polymorphism may be involved in genetic AAA susceptibility. Indeed, possessing at least an ERβ 1730A variant (*Alu*I *a* allele) was statistically associated to increased risk of AAA development. Unfortunately, the importance of ERβ 1730(A-G) polymorphism for expression and function of ERβ remains actually to be established.

Rosenkranz et al. detected a single polymorphism nucleotide exchange at nt 1730(A-G) in 3′ UTR of ERβ gene [23]. The 1730(A-G) polymorphism has presumably no functional implications, but its alleles might be in linkage disequilibrium to relevant mutations in ERβ gene. In addiction, specific control-sequence of ERα mRNA degradation pathway is located in 3′ UTR region [24]. Although no comparable data are available for ERβ gene, it might speculated that polymorphisms in 3′ UTR region of the gene could influence, e.g. mRNA stability, nuclear export, translation and post-translation process [25–26]. More studies need to address these points.

Rowe et al. identified *PROGINS* variant in the human PR gene, containing an *Alu* direct repeat insertion of PV/HS-1 *Alu* subfamily [27]. This polymorphic *Alu* insertion in PR intron 7 presents a *Taq*I restriction site detectable by PCR-RFLP. Because a consensus splice acceptor site $T_{(10)}$TGAG was identified in this *Alu* insertion, *Alu* consensus splice acceptor site potentially directs the encoding of a PR exon 8 variant with altered ligand binding and transcriptional activities [28].

Regarding clinical application of this PR gene polymorphism, we did not find any differential distribution of PR alleles between AAA patients and controls suggesting PR *Alu* element is not a genetic determinant of AAA susceptibility.

In conclusion, these findings suggest that common ERβ gene polymorphism may contribute to differential risk of AAA susceptibility. Two major considerations result from these findings. First, larger studies are clearly warranted, as well as analysis in different ethnic populations to confirm this pilot study. Second, ERβ allelic variations did not justify more than a small part of genetic susceptibility in this complex disorder suggesting other candidate genes are certainly implicated in AAA determination [28]. Probably, simultaneous investigation of a set of polymorphisms in all major genetic loci (genes' cassette) and of their reciprocal interactions in homogeneous population studies should offer a better understanding of the hereditary contribution for complex disorder such as AAA [29].

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