J Hum Genet (2005) 50:415–419
DOI 10.1007/s10038-005-0270-8

ORIGINAL ARTICLE

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# The Glu298Asp variant of the endothelial nitric oxide synthase gene is associated with an increased risk for abruptio placentae in pre-eclampsia

Received: 14 January 2005 / Accepted: 8 June 2005 / Published online: 30 July 2005


**Abstract** Attempts to define a pre-eclampsia susceptibility profile have been hampered by the wide clinical spectrum of the condition and the complex genetics underlying it. Genes that modulate blood pressure, fluid homeostasis and placental vascular development have been considered plausible candidates. Among these are the angiotensinogen (*AGT*) gene variant Met235Threo, which has been associated with pre-eclampsia and the endothelial nitric oxide synthase (*eNOS*) polymorphism Glu298Asp, which has been associated with both pre-eclampsia and abruptio placentae, a condition that often co-exists with pre-eclampsia. The aim of this study was to investigate a potential association between these gene variants and pre-eclampsia with and without abruptio placentae in a South African patient group. Fifty primigravidas with early onset, severe pre-eclampsia, 50 women presenting primarily with abruptio placentae (whether associated with pre-eclampsia or not) and a control panel of 50 healthy pregnant women constituted the study groups. The Met235Threo and Glu298Asp variants were characterised by polymerase chain reaction and restriction enzyme analysis. No association was demonstrated between the M235T variant of the *AGT* gene and pre-eclampsia or abruptio placentae. In contrast, the combined frequency of the *eNOS* variant genotypes (GT and TT) was significantly higher in the abruptio placentae group (49%) than the control group (21%) ($p = 0.006$). Furthermore, in the pre-eclampsia patients who subsequently developed abruptio placentae, the *eNOS* GT genotype emerged as a major risk factor for the development of abruptio placentae ($p < 0.0001$). These data suggest that the presence of a Glu298Asp *eNOS* variant may pre-dispose a pre-eclamptic woman to develop abruptio placentae or that it is a marker for predisposition.

**Keywords** Endothelial nitric oxide synthase · Angiotensinogen · Mutation · Pre-eclampsia · Abruptio placentae

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## Introduction

Pre-eclampsia is a form of hypertension in pregnancy that impacts significantly on maternal and foetal morbidity and mortality. The underlying cause of pre-eclampsia remains unknown despite global investigative efforts. One of the most pathologically significant clinical features of the condition is wide-spread endothelial disease characterised by vasospasm and capillary leakage. These changes can be attributed to the rennin–angiotensin system and endothelial dysfunction (Broughton 1988; Dekker and Sibai 1998).

Since the renin-angiotensin system modulates blood pressure regulation, body-fluid homeostasis and vascular remodelling in pregnancy, it is not surprising that variants in genes, such as angiotensinogen (*AGT*), encoding key components of this system have been investigated as susceptibility factors for pre-eclampsia. A transversion involving a T-C change at nucleotide position 704 (M235T) of the *AGT* gene has been implicated as a pre-eclampsia pre-disposing factor in several (Ward et al. 1993; Bernstein et al. 1998; Morgan and Ward 1999) but not all (Roberts et al. 2004) studies.

A linkage study performed by Arngrimsson et al. (1997) identified chromosomal region 7q36 [which encompasses the endothelial nitric oxide synthase (*eNOS*) gene locus] as a possible candidate region for pregnancy-induced hypertension. The *eNOS* gene product regulates blood pressure, mediates vascular dilation, affects vascular smooth-muscle proliferation and inhibits platelet aggregation, making it an attractive

candidate for pre-disposition to pre-eclampsia. Its candidacy had previously been alluded to by the report that *eNOS* mutant mice are hypertensive (Huang et al. 1995).

Several sequence variants in the *eNOS* gene have been reported, among them a single base pair substitution (G-T) at nucleotide position 894, which causes the conversion of a glutamic acid to an aspartic acid (Glu298Asp) residue (Yoshimura et al. 1998). This variant has been associated with an array of clinical conditions, including coronary spasm (Nakayama et al. 1999), emphysema (Novoradovsky et al. 1999), asthma (Grasemann et al. 2000) and essential hypertension (Jachymova et al. 2001).

Interestingly, this variant has also been implicated as a risk factor for the development of pre-eclampsia (Tempfer et al. 1999; Yoshimura et al. 2000; Hakli et al. 2003) and abruptio placentae (Yoshimura et al. 2001). One of the most unpredictable and devastating complications that can arise in pre-eclampsia is abruptio placentae, which is the premature separation of the placenta from the uterine wall. Whilst the primary cause is unknown, risk factors include chronic and pregnancy-induced hypertension, advancing maternal age, cigarette smoking and a previous history of this obstetric complication (Baron and Hill 1998; Sheiner et al. 2002).

The aim of this study was to investigate the likelihood of association between sequence variants *AGT* M235T and *eNOS* Glu298Asp in South African patients with pre-eclampsia and/or abruptio placentae.

## Patients and methods

The project was approved by the Institutional Review and Ethics board. Informed consent was obtained from all study participants. Patient and control individuals were from the South African ''Coloured'' population group who have San, Khoi, Madagascan, Javanese and European ancestry (Loubser et al. 1999). The admixture events occurred multiple generations ago and the Coloured group is an established population in South Africa.

Samples were collected from 50 control subjects (term pregnancies uncomplicated by any hypertensive condition or small for gestational age babies) and from two study groups of 50 patients each. The first group ($n = 50$) was comprised of successive primigravid patients who presented with early-onset (before 34 weeks gestation), severe pre-eclampsia and were admitted for conservative management. They were recruited at admission and followed clinically, up to delivery. The second group ($n = 50$) of patients was successively recruited after delivery for the catastrophic presenting event of abruptio placentae. During the post-delivery phase, they were observed for symptoms and signs of a possible underlying pathophysiology, and they were subsequently subgrouped into abruptio without any apparent underlying cause ($n = 20$) and abruptio with signs of underlying pre-eclampsia according to the study criteria.

Pre-eclampsia was defined as a diastolic blood pressure measurement of 90 mmHg or more measured using Korotkoff V at two occasions at least 4 h apart, coupled with significant proteinuria (2+ on diagnostic strips on at least two occasions 4 h apart, or 300 mg protein per 24-h urine sample). Early onset pre-eclampsia was recognised as onset of disease after 20 weeks but before 34 weeks of gestation. Abruptio placentae was diagnosed clinically with a sudden onset of a tender, rigid uterus, vaginal bleeding and/or foetal distress and confirmed on inspection of the placenta following delivery. A retro-placental blood clot covering more than 15% of the placental surface was required to confirm the diagnosis.

Individuals in the control panel had no hypertension, proteinuria or thromboembolic complications in the current pregnancy, nor were these present in previous pregnancies. Individuals with a history of cardiovascular or renal disease or diabetes were excluded.

DNA was extracted from whole blood collected in EDTA tubes using a GENTRA DNA isolation kit (Gentra Systems, Minnealpolis, MN, USA). The *AGT* Met235Threo polymorphism was identified by polymerase chain reaction (PCR) amplification and restriction enzyme analysis with *Asp*I (Roche, Basel, Switzerland) (Jeunemaitre et al. 1992). Oligonucleotide primers (5′-3′) F: CAG GGT GCT GTC CAC ACT GGA CCC C and R: CCG TTT GTG CAG GGC CTG GCT CTC T generated a single genomic fragment of 165 bp, which was cleaved to fragments of 141 and 24 bp in the presence of the mutant *C* allele. Restricted products were resolved on a 2% agarose gel.

The Glu298Asp variant in exon seven of the *eNOS* gene was identified by PCR amplification with oligonucleotide primers (5′-3′) F: AAGGCAGGAGACAGTGGATGGA and R: CCCAGTCAATCCCTTTGGTGCTCA. The resulting 248-bp fragment was subsequently restricted with *Ban*II (Promega, Madison, USA) and resolved on a 4% agarose gel. The wild-type G allele harbours a recognised restriction site for *Ban*II (generating fragments of 163 and 85 bp) while the mutant *A* allele abolishes the restriction enzyme recognition site (generating an undigested fragment of 248 bp) (Yoshimura et al. 1998).

The frequencies of the M235T and Glu298Asp variants among pre-eclamptic, abruptio placentae and control subjects were compared by chi-squared analysis. Odds ratios were calculated as a measure of the association between the genotypes and clinical phenotypes. For each odds ratio, $p$ values and 95% confidence intervals were calculated. A $p$ value of $< 0.05$ was considered as statistically significant.

## Results

The demographic characteristics of the participants are shown in Table 1. The abruptio group was not restricted

**Table 1** Demographic characteristics of pre-eclamptic and abruptio placentae patients and controls. Median range is shown in *parentheses*

| | Pre-eclampsia ($n=50$) | Abruptio placentae ($n=50$) | Controls ($n=50$) |
|---|---|---|---|
| Age (years) | 21 (14–31) | 28 (16–42) | 29 (18–43) |
| Primigravidas | 1 | 2 (1–6) | 3 (2–8) |
| Parity | 0 | 1 (0–5) | 2 (1–7) |
| Diastolic blood pressure at admission | 110 (80–150) | 100[a] (90–130) | 80 (60–85) |
| Gestational age at delivery (weeks) | 30 (20–36) | 33 (27–40) | 39 (37–44) |
| Birth weight (grams) | 1,260 (292–2,560) | 1,748 (762–3,702) | 3,256 (2,460–4,576) |

[a]For abruptio cases associated with pre-eclampsia. Median diastolic blood pressure on admission for patients with abruptio placentae uncomplicated by pre-eclampsia was 75 mmHg (range 60–85 mmHg)

to primigravidae only, as shown in the median gravidity. Twenty of the 50 participants in the abruptio group were primigravidae, and 30 had abruptio in association with severe pre-eclampsia (used in sub-group analysis). The median age of delivery was, as expected, lower in the early-onset pre-eclampsia group (where labour was induced for maternal or foetal complications or when a gestational age of 34 weeks was reached). This is also reflected in the lower birth weight in this group.

At the AGT M235T (T-C) locus, genotype frequencies (TT, TC and CC) and allele frequencies (T and C) were very similar in both patient groups and the control panel (Table 2). DNA analysis was not successful in three patient and five control samples.

In the analysis of the *eNOS* Glu298Asp (G-T) locus, genotyping was not successful in five patient and eight controls. Differences in genotype and allele frequencies in the pre-eclampsia and control groups were non-significant. The combined frequency of the variant in heterozygous (GT) and homozygous (TT) forms was significantly higher in the abruptio placentae group (49%) than in the control panel (21%) (OR 3.51; 95% CI 1.76–9.98; $p=0.006$). The frequency of the *eNOS T* allele was also significantly increased in the abruptio placentae group (25%) versus the control panel (8%) (OR 2.49; 95% CI 1.04–6.07; $p=0.023$). The genotypes in all groups appeared to be in Hardy–Weinberg equilibrium.

In the early onset pre-eclampsia group, three patients developed abruptio placentae during the management of their disease, and they were removed from this group. Analysis was thus performed on all the abruptio placentae cases where abruptio was associated with pre-eclampsia ($n=30$), adding the three individuals in the pre-eclampsia group who subsequently developed abruptio placentae (total $n=33$), and for comparison, analysis was also performed on the remaining pre-eclampsia patients who did not have abruptio placentae in the course of their disease ($n=46$) (Table 3).

With this analysis, the *T* allele emerged even stronger as a major risk factor for abruptio placentae in pre-eclampsia (genotype frequency: OR 5.92; 95% CI 1.85–19.65; $p=0.000$). The abruptio placentae cases associated with pathophysiological conditions other than pre-eclampsia did not demonstrate this association ($p=0.51$).

**Table 2** Genotype and allele frequencies at the *AGT* and *eNOS* loci in patient and control groups. Frequencies are shown in *parentheses*

| | Pre-eclampsia | Abruptio placentae | Control |
|---|---|---|---|
| *AGT* M235T (T-C) | | | |
| Number of alleles | 98 | 96 | 90 |
| T/T | 3 (0.03) | 4 (0.08) | 2 (0.04) |
| T/C | 10 (0.20) | 12 (0.25) | 12 (0.26) |
| C/C | 36 (0.74) | 32 (0.66) | 31 (0.68) |
| T | 16 (0.16) | 20 (0.21) | 16 (0.18) |
| C | 82 (0.84) | 76 (0.79) | 74 (0.82) |
| *eNOS* Glu298Asp (G-T) | | | |
| Number of alleles | 96 | 94 | 84 |
| G/G | 41 (0.86) | 24 (0.51) | 33 (0.78) |
| G/T | 6 (0.12) | 21 (0.45) | 8 (0.19) |
| T/T | 1 (0.02) | 2 (0.04) | 1 (0.02) |
| G | 88 (0.92) | 71 (0.75) | 74 (0.92) |
| T | 8 (0.08) | 23 (0.25) | 10 (0.08) |

**Table 3** Sub-group analysis on all patients with pre-eclampsia without abruptio placentae compared with all abruptio placentae cases associated with pre-eclampsia

| *eNOS* Glu298Asp (G-T) | Pre-eclampsia with abruptio ($n=33$) | Pre-eclampsia without abruptio ($n=46$) |
|---|---|---|
| G G | 16 (0.48) | 39 (0.85) |
| G T | 16 (0.48) | 6 (0.13) |
| T T | 1 (0.04) | 1 (0.02) |
| *G* allele | 48 | 84 |
| *T* allele | 18 | 8 |

## Discussion

In this South African Coloured patient group based in the Western Cape province, there was no evidence of an association between the M235T variant of the *AGT* gene and pre-eclampsia. This is in accordance with a recent South African study by Roberts et al. (2004). Their patients were, however, based in KwaZulu Natal province and constituted Black South African Zulu-speaking women. In this study, the presence of at least one 298Asp allele of the *eNOS* gene was identified as a significant risk factor for abruptio placentae, and strikingly so in patients with pre-eclampsia. In contrast, the variant did not seem to impact on the risk of pre-eclampsia

per se. The association of the 298Asp allele with pre-eclampsia and gestational hypertension with and without proteinuria had previously been demonstrated in two Japanese studies, respectively (Yoshimura et al. 2000; Kobashi et al. 2001), but not in others, including the recent one of Landau et al. (2004) involving an American (Caucasian and Hispanic) group. This apparent lack of association could reflect the marked difference in the distribution of the Asp298 allelic variant among different ethnic populations (Tanus-Santos et al. 2001). Our study would therefore support the lack of direct association between *eNOS* 298Asp and pre-eclampsia in this particular South African population.

In 2001, Yoshimura et al. reported the association of the 298Asp allele with placental abruption. Combined Asp heterozygote and homozygote status was reported in 40% of abruptio placentae patients ($n = 170$) versus 14% of controls ($n = 35$). These figures are remarkably similar to what was found in the present study (49% of abruptio placentae patients versus 21% of controls and 14% of pre-eclamptics). The role of the *eNOS* 298Asp allele as a susceptibility marker for abruptio placentae is hereby confirmed in a distinct population group. The homozygous mutant *eNOS* TT Asp genotype was observed rarely and at a similar frequency in the three study groups. This phenomenon could be further investigated by determining the frequency of this genotype in early gestation products of conception.

Pre-eclampsia is relatively common in the study population, with as many as one in 15 pregnancies at risk. In addition, isolated abruptio placentae is reported in ~2% of pregnancies, in 5% of pre-term labour cases and subsequently appears as a complication in ~20% of pre-eclampsia cases (Leunen et al. 2003), making this group ideal for a study such as this. While the sample numbers are relatively small in this pilot study, there is sufficient evidence and capacity to expand the study to increase sample numbers and also to stratify patients more strictly. Further stratification of patients (in terms of gestational age at onset of abruption, gravidity, extent of infarction, accompanying clinical features, etc.) may elucidate pathways that are distinct and those that are shared in pre-eclampsia and abruptio placentae.

The Yoshimura study reporting an association between the *eNOS* 298Asp allele and abruptio placentae (Yoshimura et al. 2001) stated that ten of the 35 placental abruption cases were further complicated by severe pre-eclampsia. It would be interesting to investigate the role of the *eNOS* variant in an extended group of these abruptio placentae patients further stratified into those with and those without pre-eclampsia. It would also be interesting to investigate the incidence of other 298Asp variant-associated conditions (such as coronary spasm, emphysema, asthma and essential hypertension) in the population group represented in this study and document the co-existence, if any, of these with abruptio placentae.

There is emerging evidence for a role for the 298Asp allele in abruptio placentae/pre-eclampsia as causative rather than associational. In normal pregnancy, the nitric oxide (NO) pathway becomes activated and increases NO availability. This leads to maternal vasodilation, which modulates the increase in circulating volume. Failure of this adaptation is thought to result in increased blood pressure, proteinuria, and wide-spread endothelial dysfunction (classical pre-eclampsia), arteriolar contraction, ischaemia of the decidua basalis, necrosis and haemorrhage (classical placental abruption). Although reduced NO bio-availability has already been reported in *eNOS* Asp298 homozygous individuals, there remains controversy about the interpretation of these findings (Fairchild et al. 2001; Noiri et al. 2002; Serrano et al. 2004).

At this institution (Tygerberg hospital), early onset, severe pre-eclampsia is managed expectantly, with strict maternal blood pressure control and careful foetal surveillance (Hall et al. 2000a). When abruptio placentae occurs, the outcome of birth is poor, with further complications anticipated in >50% of cases (Hall et al. 2000b). Foetal surveillance with cardiotocography is currently the best tool for early detection of abruptio placentae in these hospitalised patients, but it is not effective in predicting this pre-natal complication. Consequently, the identification of a genetic marker for abruptio placentae, such as the *eNOS* 298Glu variant, may represent is an exciting result that could impact on clinical practise.

**Acknowledgements** Unistel Medical laboratory and Mrs. D. Grove are thanked for use of equipment and statistical analysis, respectively. Sister Erika van Pappendorp is acknowledged for her endless enthusiasm and skill in recruiting patients and samples. The NRF Thuthuka program and the University of Stellenbosch are acknowledged for financial support.

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