Polymorphisms in NOS3, ACE and PAI-1 Genes and Risk of Spontaneous Recurrent Miscarriage in the Gaza Strip

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Introduction

Normal pregnancy is associated with several changes in all aspects of hemostasis. Owing to hormonal changes, increasing concentrations of procoagulants, decreased numbers of anticoagulant factors and diminished fibrinolytic activity result in pregnancy being in a hypercoagulable state in order to prevent maternal hemorrhage after delivery [1, 2]. Recurrent miscarriage (RM) is defined as 3 or more consecutive pregnancy losses before the 24th week of gestation [3]. RM may affect up to 2–4% of women of reproductive age [4].

Plasminogen activators are serine proteases which are part of an enzyme cascade converting plasminogen into plasmin. The human plasminogen activator inhibitor 1 (PAI-1) gene is located on the long arm of chromosome 7. The genomic DNA of human PAI-1 is approximately 12.2 kb long, comprising 9 exons and 8 introns [5, 6]. Urokinase plasminogen activator, its receptor, and PAI-1 are believed to control proteolysis and remodeling of maternal tissue during trophoblast invasion [7, 8]. Plasma PAI-1 concentrations have been shown to be related to a common guanosine insertion/deletion gene polymorphism, 4G/5G, 675 bp upstream from the start site of translation. Homozygosity for the deletion genotype (4G/4G) is associated with reduced fibrinolytic activity [9–13]. Glueck et al. [11] stated that homozygosity for the
4G allele of the PAI-1 gene represents a serious risk for pregnancies, predisposing to premature, intrauterine growth retardation, miscarriage, and stillbirth.

The importance of angiotensin-converting enzyme (ACE) in circulatory homeostasis is well documented and has been associated with an insertion (I)/deletion (D) polymorphism involving about 250 bp situated in intron 16 of the ACE gene, the so-called ACE I/D polymorphism [14]. Rigat et al. [15] found that the ACE I/D polymorphism was strongly associated with the level of circulating enzyme. The mean plasma ACE level of D/D subjects was about twice that of I/I subjects, with I/D subjects having intermediate levels. Fatini et al. [16] found an association of the D/D genotype with first-trimester miscarriages. Recent reports have shown that the ACE D/I polymorphism is even a stronger risk factor for RM than the two better-established thrombophilic mutations FVL and FII G20210A [17, 18].

Nitric oxide (NO) was first recognized in the reproductive system by Ignarro et al. [19]. Several polymorphic variations of the nitric oxide synthase 3 (NOS3) gene have been identified, and many of them have been studied with regard to the association with cardiovascular diseases [20]. Although some studies have demonstrated a relationship between a particular polymorphism and disease risk, many reports have been contradictory [20–22]. One polymorphism that has been examined in greater detail with respect to regulation of NOS3 expression is 27-nucleotides (5'-GAAGTCTAGACCTGCTGCAGGGTGAG-3') repeats in NOS3 intron 4 [23]. Yallampalli and Garfield [24] observed that inhibition of NO synthesis in rats during pregnancy produces hypertension, proteinuria, thrombocytopenia, and fetal growth retardation. Tempfer et al. [25] performed a prospective case-control study of 105 women with idiopathic RM and 91 healthy controls. Using PCR, they identified the different alleles of a 27-bp tandem repeat polymorphism in intron 4 of the NOS3 gene. The distribution of genotype frequencies was significantly different between the study and control groups for the A/B heterozygotes. The results obtained in the majority of studies describing genetic polymorphisms in RM are conflicting. Many of the reported associations have not been reproduced in later studies. This is partly due to the fact that the associations may vary in different ethnic populations (genetic backgrounds) and that they may be biased because of small sample sizes. One reason for a small sample size is that RM is relatively uncommon and may be as low as 1% in women.

Another concern relates to the selection of a proper control group. Some other studies investigating women with RM used age-matched controls to compare genotype frequencies [26]. This strategy did not rule out future abortions among control women. Hence the objective of this study was to investigate the association between spontaneous RM and ACE, PAI-1 and NOS3 using only postmenopausal females as controls.

### Subjects and Methods

From October 2007 to January 2008, a total of 100 women as the ‘study group’ and 100 as the ‘control group’ from different areas of the Gaza Strip, whose parents were of the same ethnicity, were included in the study. The study group consisted of women with a mean age of 28.9 years (range 20–35) who had experienced at least 3 unexplained consecutive spontaneous miscarriages before 25 weeks of gestation. All subjects were investigated ≥2 months after the last pregnancy. To exclude other factors that may increase the risk for RM, women in the study group were negative for Toxoplasma IgM, CMV IgM, Chlamydia IgM, Rubella IgM, ACL IgG or IgM and APL IgG or IgM. All study women were diagnosed as having unexplained or idiopathic RM by specialists in Alshifa Hospital or private clinics.

The control group of 100 healthy women had delivered at least one healthy, term infant and had no history of pregnancy loss. Controls were matched for ethnic background. All women in the control group were in the postmenopausal period to exclude any abortion that may occur after this study. None of the controls used oral contraceptives, hormonal, intrauterine devices, or any medication affecting liver function and/or blood coagulation.

The objective of the study was fully explained to all participants and written consent was obtained. The study was approved by the Helsinki Committee – Gaza.

#### Genotyping

Genomic DNA was isolated from blood using Wizard genomic DNA purification kit (Promega, USA), according to the manufacturer’s protocol [27].

#### Determination of ACE (I/D) Polymorphisms

The I/D genotype of the subjects was determined using PCR as described by Rigat et al. [28]. Briefly, a final volume of 20 μl consisted of 10 μl PCR Master Mix (Promega, USA), 2 μl of 10 μM forward primer (5'-CTGGGAGACCACCTCCCATCCTTTTCT-3'), 2 μl of 10 μM reverse primer (5'-GATGCGGCATCACATT-CGTCAGAT-3'), 4 μl sterile water, and 2 μl genomic DNA (approx. 30 ng/μl). The mixture was run for 35 cycles of PCR to amplify the desired segment. Each cycle consisted of denaturation at 94°C for 60 s, annealing of primers at 63°C for 90 s, and extension at 72°C for 90 s. The amplified product was electrophoresed on 1.5% DNA agarose gel, stained with ethidium bromide (10 μg/ml) and visualized using a gel documentation system (Gel Logic, USA).

#### Determination of PAI-1 (4G/5G) Polymorphisms

PAI-1 4G/5G promoter genotype was established for each subject by allele-specific PCR amplification of genomic DNA by the method of Falk et al. [29]. Two allele-specific PCRs were run per sample using 4G-specific (5'-AGAGTCTGGACACGTTGGG-
GA-3’) and 5G-specific (5’-AGAGTCTGGACACGTGGGG-3’) primers, along with a common downstream primer (5’-TGCAGACGCCAGTGTGGTAGTTCAGT-3’). An upstream primer (5’-AAGCTTTTACCATGCTAAACCCCTGGT-3’) was also used to generate an internal PCR control fragment of 257 bp. Each allele-specific primer and downstream primer amplified 136-bp bands. The allele-specific PCR mixture (20 μl final volume) contained 10 μl PCR Master Mix, 2 μl 10 μM common downstream primer, 2 μl 10 μM 4G- or 5G-specific primer, 0.8 μl 10 μM upstream control primer, 3.2 μl purified water and 2 μl purified genomic DNA (approx. 30 ng/μl). The 4G allele-specific thermal cycling conditions (32 cycles) were denaturation (at 94°C for 35 s), annealing (at 65°C for 35 s), and extension (at 72°C for 70 s). The 5G allele-specific thermal cycling conditions were identical except for the final 22 cycles which were annealed at 58°C. The amplified products were electrophoresed on 2.0% DNA agarose gel, stained at 55°C for 1 min, and extension at 72°C for 1 min. The amplified products were electrophoresed on 2.0% DNA agarose gel, stained with ethidium bromide and visualized using a gel documentation system.

**Determination of NOS3 (4a/4b) Polymorphisms**

NOS gene polymorphism in intron 4 was determined by PCR following the method of Tempfer et al. [25]. Briefly, the PCR mixture (20 μl final volume) contained 10 μl PCR Master Mix, 2 μl 10 μM forward primer (5’-AGGCCCTATGGTAGTGGCTTTT-3’), 2 μl 10 μM reverse primer (5’-TCTCTTTAGTGCTGGTGTCAC-3’), 4 μl purified water, and 2 μl purified genomic DNA (approx. 30 ng/μl). The PCR was started by preheating at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The amplified product was electrophoresed on 3.0% DNA agarose gel, stained with ethidium bromide and visualized using a gel documentation system.

**Detection of a New NOS3 Variant**

After amplification and electrophoresis of intron 4 of the NOS3 gene, an amplicon that had a size of approximately 447 bp was observed. After repeating the PCR and electrophoresis for the same sample, the same size (447 bp) was obtained. In order to understand the cause of this enlarged size, this amplicon was subjected to DNA sequencing.

**Sequencing of the New Variant**

The intron 4 PCR product was purified using the Quick Clean kit (Bioline). The sequencing reaction of the amplified fragment was conducted by asymmetric reamplification using the forward primer and dideoxynucleotides labeled with Cy5. The conditions of the reaction were as follows: denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 10 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. The products were analyzed by capillary electrophoresis on an automated ABI 3130XL genetic analyzer (Applied Biosystems).

**Statistical Analysis**

All statistical analyses were performed with SPSS v. 11.5. The differences between groups were examined by the χ² test or Fisher’s exact test, whichever was appropriate. Allele frequencies were estimated by the gene counting method. The frequencies of the alleles and genotypes were compared between study and control groups by the χ² test when appropriate. The odds ratio and 95% confidence interval were also estimated.

**Results**

**Study Population**

The number of abortions ranged from 3 to 13 with a mean of 4. Compared to the 100 controls, RM patients were younger with a high frequency of women with increasing blood pressure during pregnancy, fewer children and (by definition) more miscarriages.

**ACE Gene Polymorphism**

The results concerning the ACE polymorphism are given in table 1. The D/D genotype was less prevalent in RM patients (49%) than in controls (54%) but the difference was not significant (p = 0.479). The patients appeared to have a 1.41 times higher I/D genotype prevalence than the control subjects with a risk factor of 1.14, although there was no significant difference (p = 0.244). There was no significant difference in frequency between the genotype of the patient and control groups (p = 0.469).

**PAI Gene Polymorphism**

The genotype and allele frequencies for the PAI gene 4G/5G polymorphism in the RM patients and the controls are presented in table 2. The results show no significant difference between the groups. Unexpectedly, 4G/5G appears to be more frequent in the controls.

**NOS3 Gene Polymorphism**

The distribution of frequencies of genotypes of the NOS3 gene among RM patients and the controls is provided in table 3. The results did not show a significant difference between the two groups (p = 0.251). The 4a/4a genotype was encountered only in the RM group, although this finding was not significant. We discovered a new variant in the NOS3 gene that was named 4c allele, in 1 patient as well as in 1 control. The sequencing of the intron 4 of the NOS3 gene is shown below, with 6 repeats (underlined) of 27 nucleotides. The new NOS3 4c allele nucleotide sequences are: CTCTCTTTGGGGAGAAGCAGCACCGCCAGGCTTTTCTCCAGGAGGCTGCTCCTGCTACTGACAGCACCAGGCCAGGAGGAGCTCCTCAGAGGT; CCTCACCCCCCGCAGACCGGTCTTAGAGTT; CCTCACCCCCCGCAGACCGGTCTTAGACTT; CCTCACCCCCCGCAGACCGGTCTTAGACTT; CCTCACCCCCCGCAGACCGGTCTTAGACTT; CCTCACCCCCCGCAGACCGGTCTTAGACTT; CCTCACCCCCCGCAGACCGGTCTTAGACTT; CCTCACCCCCCGCAGACCGGTCTTAGACTT; CCTACCCCCCGCAGACCGGTCTTAGACTT; CCTACCCCCCGCAGACCGGTCTTAGACTT;
Discussion

The current study was designed to investigate the association between certain polymorphisms in PAI-1, ACE and NOS3 genes and RM in the Gaza Strip. The Palestinian population that was the subject of this investigation offered the advantage of absence of alcohol intake and cigarette smoking in women, which are the usual confounding environmental factors in such types of studies.

ACE I/D Gene Polymorphism and RM

This study determined that the D/D genotype was less prevalent in RM patients (49%) than in controls (54%), though the difference was not significant (p = 0.572). The patients appear to have a 1.41 times higher I/D genotype prevalence than the control subjects with a risk factor of 1.14; however, the difference was not significant (p = 0.244). The documented RM risk factor, the ACE D allele, was not associated with RM in the present study. It is interesting to note that the ACE D allele frequency in the population examined in this study is high relative to other reports and similar to some Arab populations [30]. In another study, patients with unexplained RM had an increased prevalence of the ACE D/D genotype compared with controls (32.1 vs. 23.6%, p = 0.11), although this was not statistically significant [31]. Our results thus suggest

### Table 1. ACE polymorphism in spontaneous RM patients and controls (100 each)

| Group | I/D | I/I | D/D | I allele frequency | D allele frequency | p value |
|-------|-----|-----|-----|-------------------|-------------------|-------|
| Patients | 42  | 9 | 49 | 60 | 140 | 0.469c |
| %     | 42.0 | 9.0 | 49.0 | 30.0 | 70.0 |       |
| Controls | 34 | 12 | 54 | 58 | 142 |       |
| %     | 34.0 | 12.0 | 54.0 | 29.0 | 71.0 |       |
| p value | 0.244b | 0.489c | 0.479d | 0.826 | 0.826 |   |

* Calculated by the χ² test. b Comparison between I/D versus I/I + D/D. c Comparison between I/I versus I/D + DD. d Comparison between D/D versus I/I + I/D. e Comparison between the patient and control groups.

### Table 2. PAI-1 polymorphism in spontaneous RM patients and controls (100 each)

| Group | 4G/5G | 5G/5G | 4G allelle | 5G allele | p value |
|-------|-------|-------|-----------|-----------|-------|
| Patients | 44 | 40 | 16 | 76 | 124 | 0.825c |
| %     | 44.0 | 40.0 | 16.0 | 76.0 | 124.0 |       |
| Controls | 48 | 36 | 16 | 80 | 120 |       |
| %     | 48.0 | 36.0 | 16.0 | 80.0 | 120.0 |       |
| p value | 0.670b | 0.662c | 1.000d | 0.682 | 0.682 |   |

* Calculated by the χ² test. b Comparison between 4G/5G versus 5G/5G + 4G/4G. c Comparison between 5G/5G versus 4G/5G + 4G/4G. d Comparison between 4G/4G versus 4G/5G + 5G/5G. e Comparison between the patient and control groups.

### Table 3. NOS3 polymorphism in spontaneous RM patients and controls (100 each)

| Group | 4b/4b | 4a/4b | 4a/4a | 4b/4c | 4b allele | 4a allele | 4c allele | p value |
|-------|-------|-------|-------|-------|-----------|-----------|-----------|-------|
| Patients | 65 | 30 | 4 | 1 | 161 | 38 | 1 | 0.251g |
| %       | 65.0 | 30.0 | 4.0 | 1.0 | 80.5 | 19 | 0.5 |       |
| Controls | 67 | 32 | 0 | 1 | 167 | 32 | 1 |       |
| %       | 67.0 | 32.0 | 0.0 | 1.0 | 83.5 | 16.0 | 0.5 |       |
| p value | 0.765b | 0.760c | 0.123d | 1.0e | 0.517a | 0.43a | 0.751a, f |       |

* Calculated by the χ² test. b Comparison between 4b/4b versus 4a/4b + 4a/4a + 4b/4c. c Comparison between 4a/4b versus 4b/4b + 4a/4a + 4b/4c. d Comparison between 4a/4a versus 4a/4b + 4b/4b + 4b/4c. e Comparison between 4b/4c versus 4a/4b + 4b/4b + 4a/4a. f Calculated by the Fisher exact test. g Comparison between the patient and control groups.
that the D allele by itself does not represent a risk factor for RM and might be considered as a heritable variant in our population.

PAI-1 4G/5G Gene Polymorphism and RM

Several studies have demonstrated that women with inherited thrombophilias carry a higher risk for RM, second-trimester abortion and other complications of pregnancy [32, 33]. This study, however, could not find a correlation between PAI-1 4G/5G polymorphism and RM. Currently we must turn our attention to observations that have been made regarding the role of PAI-1 in implantation and placentation. Several reports have pointed out that PAI-1 gene expression is critical in the first stages of pregnancy and that miscarriage may occur very early and thus goes undetectable in RM women usually enrolled in association studies.

NOS3 4a/4b Gene Polymorphism and RM

According to our results, intron 4 (4a/4b) polymorphism of the NOS3 gene is not significantly associated with RM. The 4a/4a genotype, however, may increase the risk of RM, as this genotype was observed only in the RM patients. The nonsignificant difference, however, may be the result of the small sample size enrolled in this study. Therefore, this finding deserves further in-depth investigation.

Some studies demonstrated that a lack of the gene encoding NOS3 negatively impacts early embryonic development and survival in a mouse model [34]. The association between NOS3 polymorphism and RM, as demonstrated by the present study, did not indicate significant differences but may support a functional role for NO in early embryonic development. Further studies are necessary to clarify the pathophysiological consequences of the lack of endothelium-derived NO on embryonic development. As for PAI-1, miscarriage may occur at very early stages of pregnancy.

Conclusion

Comparison between the control group and the RM patients showed that there is no significant association between ACE (I/D), PAI-1 (4G/5G) and NOS3 (4a/4b) and the occurrence of first-trimester RM. Our data indicate that in the NOS3 gene, homozygosity for 4a/4a may increase the risk of RM. Four patients showed 4a/4a as compared to the control population who did not have this genotype. The nonsignificant difference, however, may be the result of the small sample size enrolled in this study. This study also discovered a new NOS3 variant that was named 4c allele. This allele deserves further study to illustrate its effect on enzyme levels.

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