Association of Homocysteine, Asymmetric Dimethylarginine and L55M and Q192R Polymorphisms of Paraoxonase-1 Gene with Preeclampsia

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Abstract

Objective: Preeclampsia is a major cause of maternal and fetal mortality worldwide. A reliable test that would identify the "at risk" group of pregnant women is not available. Homocysteine and ADMA have been shown to be elevated in disorders characterized by endothelial dysfunction including preeclampsia. Paraoxonase 1 is thought to influence serum homocysteine and to play a role in preeclampsia.

Aim and Study Design: In this study, we aimed to measure serum homocysteine and ADMA in mild (n=24) and severe (n=12) preeclampsia compared to normotensive pregnant (n=38) and to evaluate their roles as markers to identify women at risk of developing the disease. We also investigated a possible association between PON1 55 and 192 polymorphisms and preeclampsia in Egyptian population.

Results: Homocysteine and ADMA were significantly high in preeclampsia compared to normotensive pregnancy. Both homocysteine and ADMA differed significantly between mild and severe preeclampsia. Follow-up of the normotensive pregnant women demonstrated a significant positive correlation between serum homocysteine and subsequent development of hypertension after the 36th week of gestation. No association was observed between PON1 55/192 polymorphisms and preeclampsia.

Conclusion: Our results suggest that homocysteine and ADMA may have a role in the pathogenesis of preeclampsia and could be regarded as markers for the severity of the disease. Hyperhomocysteinemia could predict women at risk of developing pregnancy-related hypertension. PON1 55/192 polymorphisms have no role in development of preeclampsia in Egyptian women.

Key Words: Preeclampsia — Homocysteine — Asymmetric — Dimethylarginine — L55M and Q192R — Polymorphisms of paraoxonase-1 gene.

Introduction

PREECLAMPSIA is a pregnancy-related multisystem disorder occurring usually after 20 weeks of gestation. Classically, it is characterized by a triad of hypertension, proteinuria, and edema, when convulsions occur in addition to these signs the condition is referred to as eclampsia. It is frequently complicated by hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome. Preeclampsia represents a significant public health threat in both developed and developing countries contributing significantly to maternal morbidity and mortality. The incidence of preeclampsia ranges between 2% and 10% of all pregnancies worldwide. WHO estimates the incidence of the disease to be seven times higher in developing countries (2.8% of live birth) than in developed ones (0.4%). The impact of the disease is felt more in developing countries, where the other causes of maternal mortality (such as sepsis and hemorrhage) are more prevalent and medical interventions may be ineffective due to late presentation of cases.

Although uterine artery Doppler studies and some maternal clinical and biochemical markers for early detection of preeclampsia have shown promise, there is not enough evidence to suggest their routine use in clinical practice. Reliable biomarkers that help to identify pregnant women at risk of developing preeclampsia before the onset of vascular abnormalities are still lacking. A possible reason for this is the uncertainty about the pathophysiology of the events leading to preeclampsia.

Preeclampsia is thought to be caused by abnormal maternal immune response to the placenta resulting in shallow placental implantation, which becomes hypoxic, leading to increased secretion of inflammatory mediators from the placenta and acting on vascular endothelium. Since many cases of the disease were shown to have normal placental implantation, it is believed that preeclampsia develop in women with higher levels of inflammation.
stems making them less tolerant to the inflammatory burden of pregnancy [6,7].

The pathophysiology of preeclampsia involves mediators of endothelial cell dysfunction. Since homocysteine has been postulated to produce oxidative stress and endothelial cell dysfunction, hyperhomocysteinemia is suggested to have a role in promoting the endothelial alterations in preeclampsia [8,9]. Homocysteine is a thiol-containing non-protein amino acid that is formed during methionine metabolism. It is produced as a product of methyl transfer reactions, which are important for methylation of nucleic acid, protein, neurotransmitters and phospholipids [10]. Under physiological conditions, homocysteine formed inside the cells is either metabolized to cysteine, or remethylated to methionine and some are exported to the blood [10,11]. Hyperhomocysteinemia is associated with decreased bioavailability of nitric oxide (NO) and increased oxidative stress leading to impairment of endothelium-dependent relaxation of blood vessels and has been recognized as an independent risk factor of hypertension [12].

The mechanism by which, homocysteine decreases NO is not fully understood [13]. Evidence is now emerging that increase of ADMA (asymmetric dimethylarginine) may be a key mediator of decreased NO in cases of hyperhomocysteinemia. Since, ADMA is produced from metabolism of methylated proteins [14]. It has been hypothesized that increased SAM-dependent protein methylation reactions which result in increased production of homocysteine would also lead to increased ADMA. ADMA has been recognized as an inhibitor of nitric oxide synthase (NOS) [15].

Several susceptibility genes were now suggested to contribute to the risk of developing preeclampsia. Human serum paraoxonase (PON1) is an enzyme bound to the high density lipoprotein HDL. It is a multifunctional antioxidant that can destroy oxidized low density lipoprotein (ox-LDL) and detoxify homocysteine metabolites [9,16]. Decreased PON1 activity has been shown to increase oxidative stress and accelerate lipid peroxidation which may lead to vascular impairment [17]. Two polymorphisms of PON1 gene have been described at positions 192 and 55, and the one at position 192 exerts greater effect on the PON1 activity [18]. PON1 gene polymorphism has been associated with cardiovascular disorders, diabetes, and preeclampsia [9,19,20].

Although some previous reports suggested a role for homocysteine and gene polymorphism in preeclampsia, others could not prove this, therefore we conducted this study to investigate serum levels of homocysteine and ADMA in preeclampsia and assess the possible association of Q192R and L55M PON1 gene polymorphism and hyperhomocysteinemia and their possible role in the pathophysiology of the disease and their possible association with the clinical severity of the disease.

**Subjects and Methods**

This study was done at the Department of Obst. and Gyn., Faculty of Medicine, Tanta University during the period from Oct. 2010-Aug. 2011. Seventy-four pregnant women were included in the study. They included 36 hypertensive pregnant women diagnosed as preeclampsia (24 patients diagnosed as mild preeclampsia and 12 were diagnosed as sever cases) and 38 normotensive, healthy pregnant women as a control group. The patients and the controls had similar distribution of age, parity, and gestational age. Preeclampsia was defined as pregnancy-induced hypertension (blood pressure 140/90 mmHg on two different occasions more than 4 h apart in a previously normotensive woman) and proteinuria 300mg in a 24 h collection urine (corresponds to 1+/2+ on a urine dipstick test) occurring after 20 weeks of gestation. Preeclampsia was considered sever when the blood pressure rises to 160/110mmHg on two different occasions at least 6h apart on bed rest and proteinuria g in a 24h collection (corresponds to 3+ or more on two random samples collected 4 or more hours apart) [21,22]. Subjects with pre-existing hypertension, cardiovascular disorders, renal diseases, diabetes mellitus and polyhydramnios were excluded from the study. Pregnant women who were smokers or vegetarians and those who had taken vitamin B12, folate, B6 suplements or antifolate drugs during the last 6 months were also excluded from the study. All women involved in the study were followed-up until delivery to assess the possible development of any complication (eclampsia or HELLP syndrome in preeclamptic women or development of hypertension in healthy women). The ethical committee of Tanta University has approved the experimental protocol. All women were included in the study after giving an informed consent. Characteristics of studied subjects are summarized in (Table 1).

Sample collection. Blood samples were obtained from all subjects and divided into two aliquots, one was used for separation of serum for homocysteine and ADMA measurement and the other aliquot was collected in EDTA (ethylene diamine tetraacetic acid) - containing tube to be used for genomic DNA extraction and genotyping of PON1 gene.
Measurement of serum homocysteine. Total homocysteine (oxidized forms, protein-bound constituting 70-80%, disulfide Hcy-Hcy 5-10%, mixed disulfide Hcy-Cys 5-10% and reduced form, free homocysteine constituting <1% [23]) was measured using the Diazyme enzymatic homocysteine assay (Diazyme Laboratories, San Diego, CA, USA). In the assay, oxidized Hcy is first reduced to free Hcy which then reacts with a co-substrate, SAM, catalyzed by a homocysteine S-methyltransferase. The co-substrate conversion product is amplified by coupled enzymatic cycling reactions. The Hcy level in the sample is indirectly proportional to the amount of NADH conversion to NAD+ (AA340 nm).

Measurement of serum ADMA. Serum ADMA was measured using ADMA ELISA kit (Biocopyre, CA, USA). The procedure was done according to the instruction provided by the manufacturer.

Genotyping of paraoxonase 55/192 polymorphism. Paraoxonase (PON1) 55/192 polymorphism was investigated using polymerase chain reaction followed by restriction digestion. Genomic DNA was extracted from whole peripheral blood using QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. The PCR amplification reactions were performed using Step-one Applied Biosystem (Applied Biosystem, USA) according to previously published protocols [18,20]. The nucleotide substitution corresponding to position 55 (Met/Leu) and 192 (Gln/Arg) creates an Hsp92II (Biogen-Fermantas) and AlwI (Biogen-Fermantas) restriction site. The PCR primers and the restriction enzymes used are listed in (Table 4).

The PCR amplification products were digested with the corresponding restriction enzyme as described (Table 2), fractionated on 3% agarose gel containing ethidium bromide, and visualized by UV illumination. Individuals homozygous for the L allele showed 384 by product and those homozygous for the M allele showed 282 and 102 by products. Regarding the Q192R polymorphism; individuals homozygous for the Q allele showed 150 by product and homozygous for the R allele showed 89 and 61 by products.

Table (1): Characteristics of studied groups.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal pregnancy (n=38)</th>
<th>Preecclampsia (n=36)</th>
<th>p-value</th>
<th>Mild preeclampsia (n=24)</th>
<th>Severe preeclampsia (n=12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29.97±6.11</td>
<td>30.97±5.56</td>
<td>0.391</td>
<td>31.33±5.41</td>
<td>30.25±6.03</td>
<td>0.574</td>
</tr>
<tr>
<td>Gestational week</td>
<td>32.08±1.496</td>
<td>33.72±1.597</td>
<td>0.72</td>
<td>34.04±1.517</td>
<td>33.08±1.621</td>
<td>0.181</td>
</tr>
<tr>
<td>Parity</td>
<td>1.76±0.786</td>
<td>1.63±0.762</td>
<td>0.78</td>
<td>1.75±0.794</td>
<td>1.417±0.669</td>
<td>0.490</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>6.09±1.81</td>
<td>10.74±3.03</td>
<td>0.001</td>
<td>9.67±2.57</td>
<td>12.86±2.82</td>
<td>0.002</td>
</tr>
<tr>
<td>ADMA</td>
<td>0.76±0.27</td>
<td>1.29±0.35</td>
<td>0.001</td>
<td>1.19±0.28</td>
<td>1.5±0.38</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Table (2): Genotypes and allele distribution of PON1 Q19R and L55M in cases and controls.

<table>
<thead>
<tr>
<th>Polymorphism genotype</th>
<th>Normal pregnancy (n=38)</th>
<th>Preecclampsia (n=36)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ</td>
<td>21 (57.9%)</td>
<td>18 (50%)</td>
<td>0.650</td>
</tr>
<tr>
<td>QR</td>
<td>12 (31.6%)</td>
<td>11 (30.5%)</td>
<td>0.924</td>
</tr>
<tr>
<td>RR</td>
<td>4 (11.1%)</td>
<td>7 (19.5%)</td>
<td>0.281</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>53</td>
<td>47</td>
<td>0.091</td>
</tr>
<tr>
<td>R</td>
<td>20</td>
<td>25</td>
<td>0.171</td>
</tr>
<tr>
<td>LL</td>
<td>19 (50%)</td>
<td>18 (50%)</td>
<td>0.999</td>
</tr>
<tr>
<td>LM</td>
<td>14 (36.8%)</td>
<td>12 (33.3%)</td>
<td>0.623</td>
</tr>
<tr>
<td>MM</td>
<td>5 (13.1%)</td>
<td>6 (16.7%)</td>
<td>0.706</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>52</td>
<td>48</td>
<td>0.895</td>
</tr>
<tr>
<td>M</td>
<td>24</td>
<td>24</td>
<td>0.996</td>
</tr>
</tbody>
</table>

Table (3): Genotypes of PON1 Q19R and L55M in mild and severe preeclampsia.

<table>
<thead>
<tr>
<th>Polymorphism genotype</th>
<th>Mild preeclampsia (n=24)</th>
<th>Severe preeclampsia (n=12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ</td>
<td>11 (45.8%)</td>
<td>6 (50%)</td>
<td>0.703</td>
</tr>
<tr>
<td>RR</td>
<td>5 (20.8%)</td>
<td>2 (16.6%)</td>
<td></td>
</tr>
<tr>
<td>QR</td>
<td>8 (33.3%)</td>
<td>4 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>LL</td>
<td>12 (54%)</td>
<td>6 (50%)</td>
<td>0.124</td>
</tr>
<tr>
<td>LM</td>
<td>8 (33.3%)</td>
<td>4 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>4 (12.5%)</td>
<td>2 (16.7%)</td>
<td></td>
</tr>
</tbody>
</table>

Table (4): The primers and restriction endonucleases used for polymorphism analysis.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Sequence of used primers</th>
<th>PCR product</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>L55M TTG/ATG</td>
<td>5'-TTGAGGAAAAAGCTCTAGTCCA-3'</td>
<td>384 by Hsp9211 (CATG)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-GGAAAGACTTAAAAGTGCCAGTCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q192R CAA/CAG</td>
<td>5'-TTGTGTGCTGTGGAGCTAGG-3'</td>
<td>150 by AIM (GGATC(N)4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5'-AATCCCTTTCTGACCACCACCTG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Statistical analysis:**

Data are expressed as mean±SD. Statistical analysis was performed with $X^2$ test, student t-test and spearman correlation. All tests were two sided and probability values <0.05 were considered significant. Analysis was done with SPSS software version 16 [24].

**Results**

The clinical characteristics of preeclamptic patients and normal pregnant women are shown in (Tables 1). No significant differences were found between the groups regarding age, parity and gestational age ($p>0.05$).

The results clearly indicate a significant elevation of plasma levels of homocysteine and ADMA among preeclampsia patients compared to the control subjects (Table 1). The mean plasma homocysteine levels in normal pregnant women were found to be 6.09±1.81 pmol/l (range; 3.7-10.3 pmol/l). In women with preeclampsia, plasma homocysteine demonstrated a significant increase (range; 7.3-17.8 gmo1/1, mean; 10.74±3.03 pmol/1, $p<0.001$) compared to normal women. Within the preeclamptic group, patients with severe preeclampsia had significantly higher plasma homocysteine (12.86±2.82 pmol/1, range; 9.6-17.8 pmol/1) compared to mild preeclampsia group (9.67±2.57 gmo1/1, range; 6.6-14.9, $p<0.002$).

Similar to the results of plasma homocysteine, plasma ADMA levels were significantly higher in preeclampsia patients (range; 0.7-2.0 pmol/1) compared to normal pregnant women (range; 0.37-1.3 pmol/1) (mean; 1.29±0.35 vs 0.76±0.27, $p<0.001$) and in severe preeclampsia patients (range; 1.0-2.1 pmol/1) compared to patients with mild preeclampsia (range; 0.7-1.6 pmol/1) (mean; 1.5±0.38 vs 1.19±0.28, $p<0.017$). No correlation was observed between plasma levels of homocysteine and ADMA in preeclampsia patients ($r=0.058, p=0.736$).

PON1 192/55 genotypes and allele frequencies were similar between healthy pregnant subjects and preeclampsia patients as shown in (Table 2) ($p>0.05$). The frequencies of PON1 192 QQ, QR and RR genotypes among the preeclampsia patients were 50%, 30.5% and 19.5% respectively and among healthy pregnant women were 57.9%, 31.6% and 11.1% respectively. Similarly, no significant difference was observed between the frequencies of PON1 55 LL, LM and MM genotypes among the preeclampsia patients (50%, 33.3% and 16.7% respectively) and controls (50%, 36.8% and 13.1% respectively). No significant difference was observed between groups as regards the alleles frequencies ($p>0.05$). No association was observed between any of the studied PON 1 genotypes and serum homocysteine and ADMA.

During the clinical and laboratory follow-up of the control pregnant women, four of them developed hypertension after the 36th week of gestation (2 of them were diagnosed as gestational hypertension, hypertension without any manifestation of proteinuria or abnormal gain of body weight, and 2 were diagnosed as preeclampsia). There was a significant positive correlation between the plasma levels of homocysteine and subsequent development of hypertension (9.0±1.1 vs 5.75±1.5, $p=0.001$). No significant correlation was observed between plasma ADMA concentrations and subsequent development of hypertension (0.95±0.34 vs 0.74±0.26, $p=0.146$).

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**Fig. (1):** Analysis of PON1 gene L55M and Q192R polymorphisms in patients and controls using PCR- RFLP. The left figure shows ponl L55M polymorphism, homozygous for L allele showed 384 by product and those homozygous for the M allele showed 282 and 102 by products. Right figure shows Q192R polymorphism; individuals homozygous for the Q allele showed 150 by product and homozygous for the R allele showed 89 and 61 by products.
Discussion

Endothelial cell dysfunction is the most popular hypothetic factor in the pathogenesis of preeclampsia [8,25]. Therefore it has been suggested that hyperhomocysteinemia may contribute to the pathogenesis and occurrence of preeclampsia.

The results of our study support the assumption of a possible linkage between plasma homocysteine and preeclampsia. A large community based study on 5883 pregnant women in Norway suggested a strong association between homocysteine and pregnancy related disorders such as preeclampsia, placental abruption, still birth and low birth weight [26].

In the present study we have clearly shown high plasma homocysteine levels in preeclamptic women. A significant direct relation has been also observed between circulating homocysteine and the severity of the disease. Our findings are in line with several previous studies that show increased maternal serum homocysteine in preeclampsia compared with normotensive pregnant women [27-30]. Supporting our data, several previous studies reported that serum homocysteine concentration can be used as an indicator of the severity of preeclampsia as well as low birth weight [27,29-32]. Singh et al. [29] reported that homocysteine levels are significantly high in preeclampsia compared with normotensive women, however these levels were comparable to those in non-pregnant women. They explained this finding by the physiological process of hemodilution in pregnancy.

Homocysteine may inhibit the activity of endothelial dimethylarginine dimethyl-aminohydrolase (DDAH) that hydrolyze ADMA, therefore hyperhomocysteinemia may be associated with accumulation of ADMA [33]. An important finding of our study was a significant increase in serum levels of ADMA in preeclampsia. There was a significant positive correlation between ADMA and the severity of preeclampsia and plasma levels of homocysteine. These data suggest that homocysteine-ADMA pathway is at least partly responsible for etiology of preeclampsia. ADMA, an endogenous nitric oxide synthase inhibitor, has been proposed as a new risk factor for endothelial dysfunction [34]. According to several studies, ADMA is increased in preeclampsia and the increase is significantly related to the severity of the disease [35-37].

The debate concerning of the potential use of blood level of homocysteine and ADMA as predictors of preeclampsia has been the subject of several reports [34,38-40]. Some studies reported that hyperhomocysteinemia in early pregnancy can be considered as a risk factor for developing preeclampsia [30-41]. In contrast, Other studies could not detect association between hyperhomocysteinemia and subsequent development of preeclampsia [42]. Regarding ADMA, it has been shown that ADMA levels may be even increased before the development of preeclampsia suggesting that ADMA might be considered as a novel marker for early detection of women at risk of preeclampsia [34,36]. According to our study, hyperhomocysteinemia but not ADMA could be considered as a risk factor for early prediction of pregnant women who are at risk of developing preeclampsia.

Hyperhomocysteinemia in preeclampsia is suggested to play a considerable role in promoting endothelial damage. Jakubowski et al. [43] reported that paraoxonase protein, carried on HDL, has homocysteine thiolactone hydrolase activity and protect against the endothelial damaging effect of homocysteine. They also reported a strong association between the paraoxonase activity and PON1 genotypes. The high paraoxonase activity was associated with L55 and R192 alleles. Therefore, they claimed that low anti-homocysteine thiolactone paraoxonase activity (M55 and Q192 alleles) combined with hyperhomocysteinemia may predispose to vascular dysfunction and preeclampsia. In contrast, Isbilen et al. [9] reported that the high paraoxonase activity (L55 and R192 alleles) was associated with elevated homocysteine in preeclampsing.

According to this study, no significant differences were found in the distribution of both PON1 L55M and Q192R genotypes as well as alleles frequencies between preeclampsia patients and normotensive pregnant women. In agreement with our PON1 genotypes results, the finding of Isbilen et al. [9]. Moreove, Lowler et al. [44] suggested that maternal R192 allele of PON1 is associated with preterm birth but not with pregnancy related hypertension. In contrast to our alleles frequency data, the findings of Yaghmaei et al. [45] who reported an association between R192 allele and risk of preeclampsia.

There are some limitations that caused differences between the current study and other studies regarding the relation between plasma homocysteine and PON1 genotype, for example, sample size, different environmental conditions, and different ethnic groups, all affecting the oxidant-antioxidant balance which in turn affect the endothelial function.
Conclusion:
To our knowledge, this is the first report on the PON1 55/192 polymorphisms combined with homocysteine and ADMA levels in preeclampsia. The present study has shown increased serum homocysteine and ADMA in preeclampsia compared to normotensive pregnant women. Homocysteine and ADMA were both associated with disease severity. No association could be detected between PON1 Q192R/L55M polymorphisms and preeclampsia. Hyperhomocysteinemia during pregnancy could be considered as a risk factor for preeclampsia.

References


