Introduction

Polycystic ovarian syndrome (PCOS) consists a heterogeneous endocrinological disorder, which is considered to be the most common cause of anovulatory infertility and hirsutism (Franks et al., 1997). It affects ~4% of unselected reproductive-aged women (Knochenhauer et al., 1998) or, according to other authors (Diamanti-Kandarakis et al., 1998; Sattar et al., 1998), 5–10% of the total population.

PCOS is characterized by oligomenorrhea, amenorrhoea, anovulation, infertility, abnormal gonadotrophins, androgen excess, hirsutism, type 2 diabetes mellitus, obesity, acne, skin hyperpigmentation and hyperinsulinaemia which is best treated with metformin (Glueck et al., 1999a, 2001). PCs is also associated with insulin-induced elevations of plasminogen activator inhibitor-1 (PAI-1). PAI-1 is the most potent inhibitor of fibrinolysis (Velasques et al., 1997; Glueck et al., 1999b) and its high levels constitute a major risk factor for miscarriage. In addition to hypofibrinolysis due to increased PAI-1 levels, miscarriages and complications in pregnancy are probably a result of prothrombotic effects of thrombophilia. It has been shown that X-linked heritable thrombophilia related to factor V Leiden (Dahlback, 1995; Bertina, 1999), heterozygosity for prothrombin factor mutation (Poort et al., 1996) and homozygosity for methylene tetrahydrofolate reductase gene (MTHFR) mutation (Arruda et al., 1997; Margaglione et al., 1998; Rozen, 2000) can cause miscarriages and serious complications in pregnancy (Kupferminc et al., 1994; Younis et al., 1997). Besides this, deficiency of protein C, protein S and antithrombin III is responsible for obstetric complications in 13% of women (Kupferminc et al., 1994).

Several studies on genetic risk factors with prothrombotic effects on pregnant or non-pregnant women have been made to assess their prevalence and the exact risk rate in different subgroups of women (Glueck et al., 2000). Thus, it was found that true risk for venous thrombosis in women who are factor V Leiden carriers and use oral contraceptives may be increased by up to 15-fold (Spannagl et al., 2000). Women with PCOS and a history of at least one pregnancy are more likely to have hereditary hypofibrinolysis with heterozygosity and homozygosity for 46/5E polymorphism of the PAI-1 gene in contrast to other women (Glueck et al., 1999b). By contrast, factor V Leiden and MTHFR mutations do not appear so frequently in women with PCOS and a history of at least one pregnancy (Glueck et al., 1999b). Recent attempts have been made to determine whether certain mutations or polymorphisms have greater prevalence among women with PCOS or are not...
linked to this genetic disorder (Urbanek et al., 1994; Franks et al., 1997).

Familial thrombophilia, for which known genetic factors have been isolated through population studies, is considered as a complex genetic disorder (Bertina, 1999). On the other hand, PCOS expression presupposes interaction of multiple factors including both genetic and environmental influences (Kahsar-Miller et al., 2001). It would be interesting to test the hypothesis that certain women with PCOS may hide a higher prevalence of mutations and polymorphisms than the general population and some of them may be the same as in familial thrombophilia. In an effort to examine this hypothesis, we studied the prevalence of antithrombin III, protein S and protein C deficiencies as well as factor V Leiden, prothrombin G20210A factor and MTHFR mutations in two different groups of women and compared the results of the group with PCOS with the group without.

Materials and methods

The study was carried out during 1998–2000 in the University Hospital of Ioannina. Thirty female patients who visited the Gynecologic Department of the Hospital seeking medical advice were diagnosed positively for PCOS and recorded by the authors. A sample of 45 women from the blood donor population of the University Hospital who did not satisfy PCOS criteria was also monitored. Clinical records included family history (especially for thromboembolic events), gynaecological/obstetric history and personal history [age, body mass index (BMI, kg/m²), smoking habits, medical drugs, operations etc.].

PCOS was diagnosed by the following criteria: (i) clinical findings (amenorrhea, oligomenorrhoea with or without history of hirsutism); (ii) hormonal test on day 3 of menstruation at 19:00–21:00 after 12 h fasting [FSH, LH, estradiol (E₂), prolactin, dehydroepiandrosterone sulphate (DHEA-S), total testosterone, sex hormone-binding globulin (SHBG), insulin and glucose] showing plasma levels at the upper limit of normal range (total testosterone 0.30–1.40 ng/ml, DHEA-S 1500–5500 ng/ml, abnormal LH/FSH ratio >2); (iii) bilaterally normal or enlarged ovaries with presence of ≥10 microcysts (2–8 mm diameter) on ultrasound examination (Adams et al., 1986).

Women with Cushing’s syndrome or hyperprolactinaemia were excluded. Women who were pregnant or had delivered during the previous 5 months, or had had a miscarriage or previous surgery during the same time period, recent myocardial infection, use of aspirin or heparin during the last 15 days, sex steroid therapy, history of haematological disease, or malignant, liver or thromboembolic disease were also excluded.

Thorough physical examination and history-taking ruled out active inflammatory, autoimmune or other disease with known effect on the mechanism of haemostasis.

Venipuncture was performed on all women on day 10 of the menstrual cycle in order to examine thrombophilic factors. Venous blood was collected on 0.129 mol/l trisodium citrate and was centrifuged twice at 2000 g for 15 min at room temperature in order to obtain plasma with relatively few remaining platelets. Plasma was then frozen and stored in small aliquots at −70°C until tested. EDTA−anticoagulant samples were used for DNA analysis. EDTA blood was snap-frozen and immediately stored at −70°C. Genomic DNA was prepared from blood samples according to standard methods.

Antithrombin and protein C activity was assessed using a chromogenic assay (Berichrom; Dade−Behring, Marburg, Germany) and total protein S was measured using a protein S clotting assay kit (Protein S; Dade−Behring). Normal ranges, obtained from 150 reference plasmas (± 2SD of the mean), for antithrombin, protein C and total protein S, were: 67–136, 68–128 and 56–127% respectively. The response of each frozen plasma to activated protein C was determined with the Pro APC kit (Dade−Behring) including a prior dilution of the patients’ plasma with factor V−deficient plasma and was expressed as the APC ratio (ratio of the clotting times obtained in the presence or absence of activated protein C). The clotting assays were performed on a BCS instrument (Dade−Behring). Normal values (± 2 SD of means) obtained from the analysis of 150 reference plasmas were 1.9–2.7 and values <1.9 were considered to demonstrate resistance to activated protein C (APC−R).

Factor V Leiden analysis

A 287 bp fragment of the factor V gene containing base pair 1691 G→A was amplified using PCR (Zoeller et al., 1994). A total of 10 μl genomic DNA was amplified in 40 μl reaction mixture [54 μl Tris–HCl, pH 8.8, 5.4 mmol/l MgCl₂, 5.4 mol/l EDTA 13.3 mmol/l (NH₄)₂SO₄, 0.8 mmol/l each dNTP, 500 μg from each primer] and 2 IU Taq polymerase by 39 cycles (94°C, 2 min; 94°C, 1 min; 58°C, 1 min; 72°C, 1 min) followed by a 5 min extension step at 72°C. Furthermore, aliquots of 16 μl of each PCR product were submitted to digestion with 2 IU of MnII for 4 h at 37°C. These aliquots were then separated by electrophoresis on ethidium bromide-stained agarose gels 2% (w/v). Digestion of the PCR products containing the wild type, heterozygous and homozygous allele with the restriction enzyme MnII results in: 37, 93, 157; 157, 130, 93, 37; and 157, 130 bp fragments respectively.

Prothrombin G20210A analysis

A 345 bp fragment of the prothrombin gene containing base pair 20210 G→A was amplified using PCR (Poort et al., 1996). A total of 40 μl genomic DNA was amplified in 40 μl reaction mixture [54 μl Tris–HCl, pH 8.8, 5.4 mmol/l MgCl₂, 5.4 mol/l EDTA 13.3 mmol/l (NH₄)₂SO₄, 0.8 mmol/l each dNTP, 500 μg from each primer] using 2 IU Taq polymerase for 39 cycles (94°C, 2 min; 94°C, 1 min; 58°C, 1 min; 72°C, 1 min) followed by a 5 min incubation at 72°C. Furthermore, aliquots of 16 μl of each PCR product were submitted to digestion with 2 IU of HindIII for 2 h at 37°C. These aliquots were then separated by electrophoresis on ethidium bromide-stained agarose gels 2% (w/v). Digestion of the PCR products containing the wild type, heterozygous and homozygous allele with the restriction enzyme HindIII results in: 345; 345, 322, 23; and 322, 23 bp fragments respectively.

MTHFR analysis

A 198 bp fragment of the MTHFR gene containing base pair 677 C→T was amplified using PCR (Frost et al., 1995). A total of 10 μl genomic DNA was amplified in 40 μl reaction mixture [50 μl Tris–HCl, pH 8.8, 5 mmol/l MgCl₂, 10 mol/l EDTA 13.3 mmol/l (NH₄)₂SO₄, 0.8 mmol/l each dNTP, 250 μg from each primer] using 2 IU Taq polymerase for 39 cycles (94°C, 2 min; 94°C, 1 min; 58°C, 1 min; 72°C, 1 min) followed by 5 min at 72°C. Furthermore, aliquots of 16 μl of each PCR product were submitted to digestion with 2 IU of HinfI for 2 h at 37°C. These aliquots were then separated by electrophoresis on ethidium bromide-stained 2% agarose gels. Digestion of the PCR products containing the wild type, heterozygous and homozygous allele with the restriction enzyme HinfI results in: 345; 345, 322, 23; and 322, 23 bp fragments respectively.

Statistical analysis

In order to compare the hormonal profile of the two samples we measured DHEA-S, E₂, SHBG, glucose, insulin, prolactin, LH and
FSH in women’s serum and calculated means with SD for each hormone. For quantitative variables, assessment for normality of the spread was carried out by using a Kolmogorov–Smirnov test. Equality of variances was assessed by an F-test. We used Student’s t-test to compare mean age and mean BMI of the two samples as well as mean hormonal values. We also applied Fisher’s exact test to assess smoking habits and parity status. In addition, we measured median activity of protein S, protein C and antithrombin III. Finally, we estimated the odds ratio (OR) for mutation prevalence of MTHFR, factor V Leiden and prothrombin G20210A factor between the two samples and also applied a $\chi^2$ test where possible. All $P$-values are two-tailed. $P = 0.05$ was accepted as statistically significant.

**Results**

We recorded 30 women with verified PCOS. The mean (SD) age of women was 22.57 (2.43) years. The sample of women without PCOS consisted of 45 women who had a mean (SD) age of 22.40 (2.48) years. All women were in good health, euthyroid, with normal glomerular filtration rate and negative family history for thromboembolic events. They also had BMI within the normal range (21–27 kg/m²). Mean (SD) BMI was 24.2 (1.75) kg/m² in women with PCOS and 24.1 (1.55) kg/m² in non-PCOS women. All 30 women with PCOS were nulliparous and six (20%) were smokers; 28 (62.2%) women without PCOS were nulliparous and 19 (42.2%) were smokers. There was a significant difference in smoking habits ($P = 0.05$) as well as nulliparity ($P < 0.001$) between the two samples. The characteristics of the two samples are shown in Table I.

Table II shows mean (SD) hormonal values. Among women with PCOS, 18 (60%) patients were found to have testosterone levels higher than normal, seven (23%) had higher levels of DHEA-S and five (16.7%) women an LH:FSH ratio > 2. The levels of glucose, insulin, SHBG, prolactin and $E_2$ were all in the normal range. The differences observed between the two samples were statistically significant for $E_2$, SHBG, prolactin, FSH, testosterone ($P < 0.001$), insulin ($P = 0.001$) and LH ($P = 0.018$) whereas there was no significant difference for DHEA-S and glucose. Both groups followed a normal distribution for all the quantitative variables. Although there was significant difference in insulin levels, no sample showed increased insulin resistance, since all values were in normal range.

Ultrasound images corresponding to polycystic ovaries were found in 21 (70%) of PCOS women. Of these, 23.8% (5/21) had an LH:FSH ratio > 2, 47.6% (10/21) revealed testosterone levels higher than normal and 28.6% (6/21) had DHEA-S levels higher than normal.

Thrombophilia profile in the 30 women with PCOS showed a wild type for factor V Leiden and prothrombin G20210A mutation. Four (13.4%) were homozygous for MTHFR mutation, 14/30 (46.6%) were heterozygous and 12/30 (40%) had the wild type for MTHFR. All 30 women showed normal median proportions (ranges) for activated protein S: 87.20 (54.60–112.50); C: 93.80 (83.74–129.05); and antithrombin III: 97.10 (66.38–126.79), as presented in Table III.

In non-PCOS women, prevalence of MTHFR homozygosity, heterozygosity and wild type was 6/45 (13.3%), 19/45 (42.2%) and 20/45 (44.5%) respectively. In addition, all 45 women showed a wild type distribution for factor V Leiden and prothrombin G20210A. They also had normal median proportions (ranges) for activated protein S: 91.50 (65.10–118.00); protein C: 94.20 (87.10–127.2); and antithrombin III: 98.30 (69.10–124.70), as presented in Table III.

As shown in Table IV, the OR for bearing a mutation on the MTHFR gene is 1.2-fold higher [95% confidence interval (CI): 0.470–3.065] in women with PCOS than in women without PCOS. Although this difference is far from statistically significant ($P = 0.813$), it may imply a higher prevalence of heterozygous genotype in women with PCOS (OR: 1.197, 95% CI: 0.473–3.034). On the contrary, there was absolutely

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**Table I. Characteristics of the two studied groups**

<table>
<thead>
<tr>
<th></th>
<th>PCOS ($n = 30$)</th>
<th>Non-PCOS ($n = 45$)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.57 (2.43)</td>
<td>22.40 (2.48)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.20 (1.75)</td>
<td>24.06 (1.55)</td>
<td>NS</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>6 (20)</td>
<td>19 (42.2)</td>
<td>0.050</td>
</tr>
<tr>
<td>Nulliparous, n (%)</td>
<td>30 (100)</td>
<td>28 (62.2)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean (SD).

PCOS = polycystic ovarian syndrome; NS = not significant.

**Table II. Hormonal values of the two study groups**

<table>
<thead>
<tr>
<th></th>
<th>PCOS ($n = 30$)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Non-PCOS ($n = 45$)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Normal values</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA-S (ng/ml)</td>
<td>3261.10 (1624.71)</td>
<td>2791.84 (1.55)</td>
<td>1500.00–5000.00</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol (µmol/l)</td>
<td>258.41 (69.83)</td>
<td>172.13 (104.23)</td>
<td>37.00–330.00</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>29.9 (12.28)</td>
<td>42.45 (15.71)</td>
<td>13.00–70.00</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>92.17 (12.07)</td>
<td>93.50 (7.89)</td>
<td>70.00–125.00</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>17.63 (3.65)</td>
<td>14.15 (4.50)</td>
<td>6.00–24.00</td>
<td>0.001</td>
</tr>
<tr>
<td>Prolactin</td>
<td>15.53 (4.71)</td>
<td>20.11 (3.60)</td>
<td>0.10–25.00</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>7.87 (2.01)</td>
<td>5.70 (2.00)</td>
<td>2.00–10.00</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>7.47 (2.66)</td>
<td>9.16 (3.10)</td>
<td>0.00–14.00</td>
<td>0.018</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>0.95 (0.39)</td>
<td>0.64 (0.21)</td>
<td>0.30–1.40</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean (SD);

PCOS = polycystic ovarian syndrome; DHEA-S = dehydroepiandrosterone sulphate; SHBG = sex hormone-binding globulin; NS = not significant.
no evidence that the genetic analysis for factor V Leiden or prothrombin factor differed between the two samples, since no such mutations were found.

Discussion
Inherited thrombophilia is a group of genetic disorders of blood coagulation, which results in increased risk for thrombosis. Several studies during the last 3 years have suggested that not only should they be regarded as disorders associated with increased risk for thromboembolic disease during pregnancy and post-partum, but they should also be associated with increased incidence of miscarriages (Brenner, 1999; Rosendaal, 1999). Factor V Leiden, prothrombin factor G20210A polymorphism and MTHFR (C677T) mutation account for the majority of thromboembolic events, particularly during gestation or in association with oral contraceptive use (Foka et al., 2000; Rai et al., 2000, 2001; Rozen, 2000). APC resistance due to factor V Leiden is related to a slightly increased risk for thrombosis in heterozygous carriers and to a greatly increased risk in homozygous individuals. On the other hand, APC-resistant women have reduced bleeding tendency after delivery, which may explain survival of this genotype and higher prevalence of the corresponding mutation (5–15%) (Dahlback, 2000).

Previous studies show that women with PCOS did not have an increased prevalence of APC resistance, which is caused by a point mutation in the V gene factor (known as V Leiden), compared with women in general population (Atiomo et al., 2000). These results agree with ours and suggest that APC resistance most probably does not play a role in PCOS. Prevalence of V Leiden varies among different countries in Europe, ranging from 2% in southern countries to 10–15% in northern countries (Dahlback 1995, 2000). In Greece the prevalence is 4.3% (Lambropoulos et al., 1997).

The second most common genetic risk factor for thrombosis, single mutation (G20210A), is found in ~2% of the white population and in 2.8% of the Greek population (Poort et al., 1996; Hillarp et al., 1997; Foka et al., 2000). No such mutation was found in either group in our study.

Heterozygous deficiencies of protein C, protein S and antithrombin III increase the risk for thrombosis (1–2% in patients with thrombosis) but are uncommon in the general population (1:200–1:300 for protein C, in voluntary blood donors). Frequency for antithrombin III heredity is ~1:5000 in the general population (Vinazzer 1999; Bertina 2000). In our study, we found no women in the two groups with protein S, protein C or antithrombin III deficiency. Heterozygous deficiency of protein C may be difficult to diagnose given the inherent biological variability and wide normal ranges in functional test systems. Heterozygous and normal individuals may overlap at the lower end of the normal reference range (Nizzi and Kaplan, 1999).

Heterozygous carriers for MTHFR mutation have been estimated at 30–40% in the population (Schneider et al., 1998), in accordance with our results. Heterozygosity for MTHFR factor, which causes a 50% reduction in enzyme activation, is related to moderate hyperhomocysteinaemia or to normal homocysteine levels regardless of folate status (DeStefano et al., 2000).

Homoyzogous genotype of C677T MTHFR mutation is associated with hyperhomocysteinaemia in the presence of low folate concentration. Prevalence of homoyzogous persons in different races is 13.9% (DeStefano et al., 2000; Rozen 2000). According to our study, women with PCOS had a prevalence of 13.3%, as did women without PCOS. Prevalence in Europeans is 24–40%, in Japanese populations 26–37% and in African-American populations 11% (Schneider et al., 1998). The high frequency of the C677T mutation worldwide is surprising if homozygotes have an increased risk of disease.

### Table III. Activated protein proportions and activated protein C ratio (APCR) in the two studied groups

<table>
<thead>
<tr>
<th></th>
<th>PCOS (n = 30)</th>
<th>Non-PCOS (n = 45)</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein S (%)</td>
<td>87.20 (54.60–112.50)</td>
<td>91.50 (65.10–118.00)</td>
<td>56.00–127.00</td>
</tr>
<tr>
<td>Protein C (%)</td>
<td>93.80 (83.74–129.05)</td>
<td>94.20 (87.10–127.2)</td>
<td>68.00–128.00</td>
</tr>
<tr>
<td>Antithrombin III (%)</td>
<td>97.10 (66.38–126.79)</td>
<td>98.30 (69.10–124.70)</td>
<td>67.00–136.00</td>
</tr>
<tr>
<td>APCR</td>
<td>2.30 (1.90–2.50)</td>
<td>2.40 (1.80–2.50)</td>
<td>1.9–2.7</td>
</tr>
</tbody>
</table>

*aMedian (range).*  
PCOS = polycystic ovarian syndrome.

### Table IV. MTHFR mutation prevalence in the two studied groups

<table>
<thead>
<tr>
<th></th>
<th>PCOS (n = 30)a</th>
<th>Non-PCOS (n = 45)a</th>
<th>Odds ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous MTHFR</td>
<td>4 (13.3)</td>
<td>6 (13.3)</td>
<td>1.000 (0.257–3.892)</td>
<td>1.000</td>
</tr>
<tr>
<td>Heterozygous MTHFR</td>
<td>14 (46.7)</td>
<td>19 (42.2)</td>
<td>1.197 (0.473–3.034)</td>
<td>0.813</td>
</tr>
<tr>
<td>Total</td>
<td>18 (60.0)</td>
<td>25 (55.5)</td>
<td>1.200 (0.470–3.065)</td>
<td>0.813</td>
</tr>
</tbody>
</table>

*aValues in parentheses are percentages.*  
PCOS = polycystic ovarian syndrome; CI = confidence interval.
One possible explanation is that either heterozygous or homozygous mutant genotypes may, in certain circumstances, have a selective advantage over normal individuals. Two such theories have been suggested: a decreased risk of C677T homozygotes for colon cancer (Chen et al., 1996) and a beneficial effect for heterozygotes during times of starvation (Engbersen et al., 1995). In the second hypothesis, the thermolabile form of MTHFR factor is believed to decrease homocysteine remethylation so that the 1-carbon moieties of derivatives remain available for the vital synthesis of purines and thymidine.

Most available studies do not suggest that a higher risk exists for thrombosis in individuals with inherited thrombophilia and C677T MTHFR homozygous genotype as detected by means of genetic printing, without considering homocysteine levels (Willems et al., 2000). In most cases, homocysteine concentration is probably the result of an interaction among different factors, such as pregnancy, drugs, etc. (Guba et al., 1999). For example, metformin, which is used successfully in women with PCOS, increases homocysteine levels (Snedece et al., 2000) and its consequences would be interesting to study when the homozygous MTHFR genotype coexists. This would be particularly interesting in pregnant women with PCOS, for whom metformin appears to be an effective and safe drug (Glueck et al., 2001). On the contrary, homocysteine is reduced by sex hormones in women during pregnancy (Guba et al., 1999).

This study had some statistical limitations because of the small sample size. However, it may serve as a pilot study from which the sample size required for a future definitive study could be calculated. What makes our sample interesting is the lack of thrombotic risk factors even in women with PCOS, such as family history of thromboembolic events, insulin resistance, obesity and excessive smoking habits. Nevertheless, the size in each group would need to be 4–6-fold larger in order to have a power of 80%, considering the frequencies of molecular risk factors as they have been defined in general population, in previous studies.

In conclusion, molecular risk factors of hereditary thrombophilia such as factor V Leiden, prothrombin factor, protein S, protein C and antithrombin III do not appear with increased prevalence in women with PCOS in comparison with women in the general population. The insignificant observation of slightly higher prevalence of MTHFR mutation in women with PCOS needs further study, particularly regarding homocysteine levels. Women with PCOS seem to be able to follow hormonal therapy without having increased risk for thromboembolic complications when the same principles as in non-PCOS women are applied. The increased prevalence of pregnancy complications in women with PCOS must be attributed to mechanisms other than haemostasis malfunction caused by the factors studied.

Acknowledgement
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