Modulation of Factor V Levels in Plasma by Polymorphisms in the C2 Domain

Daniela Scanavini, Domenico Girelli, Barbara Lunghi, Nicola Martinelli, Cristina Legnani, Mirko Pinotti, Gualtiero Palareti, Francesco Bernardi

Objective—Functional polymorphisms contributing to coagulation factor levels are preferential markers for association studies aimed at identifying prothrombic genetic components.

Methods and Results—Factor V (FV) microsatellite genotypes were found to be associated with FV levels \( (P=0.003) \). Single nucleotide polymorphisms analysis and sequencing of the promoter and of coding regions identified two polymorphisms (Met2120Thr, Asp2194Gly) present in 20% of the population \( (n=1013) \) that are responsible for genotype-phenotype associations. The effect of the Met2120Thr polymorphism, both in plasma (mean reduction of FV level in the heterozygous condition: 25%) and in recombinant FV studies (34% reduction), was comparable to that of the Asp2194Gly change (20% and 34%, respectively). The study of 10 subjects with a rare genotype indicated that the Asp2194Gly substitution is the functional determinant of the reduced FV levels associated with the FVHR2 haplotype. Among Leiden carriers, the doubly heterozygous condition for FV2120Thr was found to be associated with a significantly increased activated protein-C resistance (APCR) \( (P<0.05) \), and the doubly heterozygous condition for FV2194Gly was found to be more frequent \( (P=0.009) \) in symptomatic than in asymptomatic subjects.

Conclusions—Extensive analysis of FV polymorphisms indicated that changes in the C2 domain modulate FV levels and might increase APCR and thrombotic risk in FV Leiden carriers through a pseudohomozygous mechanism. (Arterioscler Thromb Vasc Biol. 2004;24:1-8.)

Key Words: XXXXX • XXXXX • XXXXX • XXXXX • XXXXX • XXXXX

Polymorphisms contributing to modulate factor levels might constitute preferential markers for association studies aimed at identifying prothrombic or protective genetic components.1–3 Among the coagulation factors, factor V (FV)4 plays a pivotal role at the crossroads of procoagulant and anticoagulant pathways,5 acting as a cofactor in prothrombin activation and in activated factor VIII (FVIII) inactivation.6 A complex relationship between plasmatic FV levels and thrombosis might be present; high FV coagulant levels have been found to be an independent risk factor for myocardial infarction.7 Whereas no significant association was found between FV antigen level and thrombotic risk.8 Moreover, low FV levels are associated with a reduced activated protein-C (APC) cofactor activity in the inactivation of the FVIIIa,9–11 which could be responsible for an APC-resistant phenotype. In particular, low FV levels in carriers of FV Leiden mutation result in a pseudohomozygous state of APCR resistance (APCR).12

Although FV genetic components of APCR have been extensively investigated,13–18 no systematic analysis of the relationship between FV gene variation19 and FV levels8 has been conducted. The His1299Arg polymorphism,20 which marks a FV gene haplotype (FVHR2) predicting several amino acid substitutions21,22 in the A2, B, A3, and C2 domains,23,24 has been associated with reduced FV activity20 and antigen25 levels. Because the increased risk for venous thrombosis26 conferred by the FVHR2 haplotype is still a matter of debate,25,27–31 markers of this haplotype are currently investigated in several laboratories.

FV expression studies have indicated that the Asp2194Gly change in the C2 domain, among the several polymorphisms of the FVHR2 haplotype, is responsible for significantly reduced FV levels32,33 in conditioned media and for reduced secretion rate.32 Linkage of markers in the haplotype22 has prevented validation in vivo of this functional candidate.

We investigated, in a large cohort of subjects, the presence of frequent genetic components of plasma FV levels. The relationship of candidate single nucleotide polymorphisms (SNPs) with APCR and venous thrombosis was then evaluated in FV Leiden carriers. Finally, the functional consequences of SNPs were investigated through expression of recombinant FV.
FV gene localization of the microsatellite and SNPs investigated in this study. Selected exons are reported together with nucleotide and amino acid changes. Polymorphisms modulating FV levels are reported in bold. The alleles characterized the FVR2 are indicated by an asterisk.

Methods

Population Studies
In this study, 1013 subjects (769 males and 244 females, mean age: 60.1 ± 10.6 years) living in Verona were enrolled in the frame of a regional cardiovascular disease survey, characterized for FV levels, and genotyped for FV polymorphisms.

Of these subjects, 122 carriers of FV Leiden mutation (42 males and 80 females, mean age at the first thrombotic event: 35.9 ± 12.8 years) were characterized for APC ratio and genotyped for FV polymorphisms. They were recruited in a thrombophilia screening and met the following criteria: (1) objectively confirmed deep venous thrombosis and/or pulmonary embolism; (2) blood sampled >3 months after the last thrombotic event and >3 weeks after anticoagulation withdrawal; and (3) normal liver function and no evidence of autoimmune or neoplastic disease.

Two-hundred ninety unrelated carriers with FV Leiden mutation diagnosed between September 1994 and January 2001 in the Angiology Department of Bologna University were investigated for the association of FV polymorphisms with venous thrombosis. Subjects were selected and matched as previously reported. All subjects were of Italian origin and none had other known thrombophilic conditions (such as antithrombin, protein C and protein S deficiency, or G20210A mutation of the prothrombin gene). Of these subjects, 145 were unrelated patients who experienced an objectively confirmed venous thromboembolic episode (VTE) (49 males and 96 females, mean age at first VTE: 35.2 ± 11.6 years). The 145 unrelated subjects were still asymptomatic for VTE at presentation (49 males and 96 females, mean age at presentation: 52.1 ± 12.0 years) and were investigated for the presence of a family history of VTE or screened before they were administered oral contraceptive pill or hormone replacement therapy.

Either written or verbal informed consent was obtained from all subjects. The study was performed according to the Helsinki protocol and approved by our institutional review board.

DNA Studies
FV markers investigated in this study are shown in Figure 1 (numbering in accordance with that of Jenny et al). Automated sequencing was performed with the ABI Prism 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA). Primer sequences and polymerase chain reaction (PCR) conditions are listed in Table I (available online at http://atvb.ahajournals.org).

The FIVS3 primer and a mutagenized reverse primer (5'-GCATGTTCAATTCTAGTAAAGTA-3'), introducing a Mbol restriction site in the wild-type (G) allele. Genotyping for the FV 1691A/G (Arg506Gln, 4070A/G (His1299Arg), 5380A/G (Met1736Val) polymorphisms, and for the intron 11 microsatellite marker was performed as previously reported. The C/A polymorphism was in the intron 22 (−20 bp from exon 23; nt 31701 at accession number Z99572) was identified by HpyCH4IV digestion of the PCR fragments FIVS23–RIVS23. The 6533T/C (Met2120Thr) change was screened by amplification of a 316-bp fragment with the primers FIVS24–RIVS24, followed by HpyCH4IV digestion.

A mutagenized primer (5'-CGTAATGTTCAATTCTAGTAAAGTA-3'), introducing a Rsal restriction site in the rare (G) allele, was designed to detect the 6755A/G (Asp2194Gly) change. A multiplex PCR (30 seconds at 95°C, 30 seconds at 56°C, 1 minute at 72°C, 30 cycles) was set-up for the analysis of His1299Arg and Asp2194Gly polymorphisms, which were both detectable through Rsal digestion.

FV Expression
The pMT2-V expression vector containing the full-length human FV cDNA was used as a template for site-directed mutagenesis (QuickChange system; Stratagene, La Jolla, CA). The Met2120Thr and Asp2194Gly changes were obtained by the mutagenic primers (forward sequence) 5'-CTCTGGTCCTCCTGAAAACGTATGTAAGAGCTTATCC-3' and 5'-GAACTCTTTGGCTGTTGTATGATTTACTAGAATTGA-3', respectively. The presence of the mutations was confirmed by DNA sequencing. Wild-type and mutant vectors (5 μg) were transiently transfected in COS-7 cells (5 × 10⁴ cells) in serum-free medium using FuGene 6 (Roche Diagnostics, Indianapolis, IN). Different preparations of each vector were used, and the transfection efficiency was evaluated by co-transfection with pGL3 vector (1 μg) expressing the luciferase reporter gene (Promega, Madison, WI). Conditioned media were harvested 72 hours after transfection and stored at −80°C.

FV Activity
Plasma FV activity was measured by a one-stage clotting method (Tromborel S and FV depleted plasma, Dade Behring, Marburg, Germany). APCR was measured in accordance with the methods of de Ronde and Bertina.

The activity of recombinant FV molecules was evaluated as thrombin generation in a FV depleted plasma system diluted 1:6 in medium containing recombinant FV and incubated at 37°C for 3 minutes with Innovin (Dade Behring) to trigger coagulation. Aliquots of the reaction were then quenched with 12 mmol/L EDTA. Thrombin was quantified by estimating its activity toward 250 μmol/L thrombin fibrinogenic substrate (ICN Biomedicals, Costa Mesa, CA). Fluorescence (360 nm excitation, 465 nm emission) was monitored over time on a Spectrafluor Plus microplate reader (Tecan, Salzburg, Austria). The initial rates, expressed as relative fluorescence units (RFU) per second, were derived from thrombin generation curves. Rates obtained for mutant proteins were compared with those of the wild-type protein by Student’s t test, and the activity of mutants was expressed as percentage of wild type.

FV Antigen
FV antigen was determined using a two-site immunosassay (ZYMUTEST Factor V kit; HYPHEN BioMed, Andrésy, France).

Statistical Analysis
Statistical analyses were performed with SPSS 10.0 statistical package (SPSS, Chicago, IL). FV activity distributions in subjects grouped for genotypes were compared by the ANOVA with Bonferroni post-hoc comparison of the means. Differences between recombinant FV levels were assessed by Student’s t test. FV polymorphism distributions in FV Leiden carriers were compared in
Hardy–Weinberg equilibrium of genotype frequencies was also found to hold in coding regions. SNPs Analysis

SNPs in the 15R homozygotes (n=38) indicated that the 5380A/G change (Met1736Val) in the exon 16 was over-represented (22/38 “GG” homozygotes; G allele frequency: 75%) among these subjects. As comparison, the G allele frequency in the whole population was 40%. However, no significant differences in FV levels were observed in subject groups for IVS11 genotype combinations (see Table 1).

The Asp2194Gly polymorphism in the C2 domain, which is suggested as a functional candidate of the FVHR2 haplotype by expression studies, was further investigated. To favor the detection of the rare subjects characterized by the FVHR2 haplotype with a wild-type (2194Asp) C2 domain, genotyping was extended to a larger sample (1013 subjects; Table 2), which included the 402 subjects investigated in this study. A multiplex PCR system was designed to detect both His1299Arg and Asp2194Gly polymorphisms. One hundred thirty-four subjects heterozygous for the His1299Arg change were also carriers of the Asp2194Gly change. However, 10 subjects were found to be homozygous for Asp at position 2194 and thus were carriers of a FVHR2 variant. In this FV gene, linkage disequilibrium between the 1299Arg and the 2194Gly codons located in the exon 13 and 25, respectively, has been interrupted by recombination, as supported by genotyping for the −20 C/A polymorphism in intron 22.
TABLE 2.  FV Activity Distribution in Subjects Grouped by SNP Genotypes

<table>
<thead>
<tr>
<th>FV Genotypes</th>
<th>n</th>
<th>%</th>
<th>FV Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Wt/Wt</td>
<td>812</td>
<td>80.2</td>
<td>139.5±38.3</td>
</tr>
<tr>
<td>2 Wt/HR2v</td>
<td>10</td>
<td>1.0</td>
<td>141.6±38.0</td>
</tr>
<tr>
<td>3Wt/HR2</td>
<td>134</td>
<td>13.2</td>
<td>113.3±34.3*</td>
</tr>
<tr>
<td>4Wt/2120Thr</td>
<td>45</td>
<td>4.4</td>
<td>107.8±31.3*</td>
</tr>
<tr>
<td>5 HR2/HR2</td>
<td>8</td>
<td>0.8</td>
<td>95.9±22.7*</td>
</tr>
<tr>
<td>6 2120Thr/2120Thr</td>
<td>1</td>
<td>0.1</td>
<td>136.0</td>
</tr>
<tr>
<td>7 2120Thr/HR2</td>
<td>3</td>
<td>0.3</td>
<td>93.0±8.0</td>
</tr>
<tr>
<td>All genotypes</td>
<td>1013</td>
<td>100</td>
<td>134.2±39.0</td>
</tr>
</tbody>
</table>

FV haplotypes are separated by a slash.
Wt indicates haplotype 1299His 2120Met 2194Asp; HR2v (variant), 1299Arg 2120Met 2194Asp; HR2, haplotype 1299Arg 2120Met 2194Gly; 2120Thr, haplotype 1299His 2120Thr 2194Asp.

n and % indicate number and percentage of subjects, respectively.

FV activity was measured as % of positive-negative-positive (mean ± SD).

ANOVA (F = 22.31, P < 0.0001) was conducted for genotypes present in at least 8 subjects.

*Significantly different from the Wt group (Bonferroni test, P < 0.05)

We found that the A allele belongs to the FVHR2 haplotype (unpublished observation). The 1299HisArg-2194AspAsp subjects (n=10) were found to be homozygous for the CC (wild-type) genotype, showing that the recombination occurred downstream to IVS22.

The 1299HisArg-2194AspAsp genotypes offer the opportunity to test the contribution in plasma of the Asp2194Gly change to the FV phenotype. FV activity (Table 2) was significantly (P < 0.05) lower in the 1299HisArg-2194AspGly subjects (n=134) than in homozygotes for the wild-type FV gene (1299HisHis-2194AspAsp, n=812), whereas the rare (n=8) 1299HisArg-2194AspAsp subjects had FV activity similar to that of the wild-type group.

Search of New Determinants by Nucleotide Sequencing

When the 2194Gly carriers distributed among the genotype groups in Table 1 (70 of 402, mean FV:C 110.9±26.3 SD) were excluded from the ANOVA, significant differences in FV levels were no longer detectable (F=0.73; P=0.664). This finding indicates that the Asp2194Gly change was the main genetic component underlying FV level differences detected by the microsatellite marker, thus preventing further genetic studies based on this approach.

To find new genetic components of FV levels not identified by the microsatellite analysis, 20 subjects with the lowest FV levels (first and second percentiles, FV:C ≤71%) in the absence of the Asp2194Gly polymorphism were further investigated in a wide part (80%) of the coding region and in the 5’ portion. A total of 5.4 kb of FV genomic DNA was sequenced using the PCR conditions and the primers reported in Table I. The Met2120Thr polymorphism was detected in 5 subjects, whereas the Tyr1702Cys mutation, previously found to be responsible for FV deficiency, was detected in 1 subject. Both mutations were present in the heterozygous condition.

All subjects (Table 2) were then genotyped for the Met2120Thr marker. Carriers of the 2120Thr allele (n=45) showed a significant (P<0.05) decrease in FV activity, comparable to that observed in 2194Gly carriers (n=134). Doubly heterozygotes (n=3) showed the lowest FV levels, comparable to those of the 2194Gly homozygotes (n=8).

Further genotyping showed that the Met2120Thr change was associated with the 14R allele, which marks the most frequent FV haplotype in our population. This observation explains why the microsatellite marker analysis (Table 1) was not useful to highlight the influence of the Met2120Thr polymorphism.

The Asp79His change recently reported to modulate FV activity (36) was detected in 5 subjects by sequencing of the exon 3. Screening for this polymorphism was extended to the whole population. We did not find significant differences (ANOVA, F = 2.10, P = 0.123) in FV levels among genotype groups: 79Asp/Asp (Wt) (genotypic frequency 88%; mean FV:C 135.1±38.5 SD), 79Asp/His (genotypic frequency 11.7%, mean FV:C 127.8±42.9 SD) and 79His/His (genotypic frequency 0.3%, mean FV:C 117.0±34.0 SD). This marker was not further investigated.

Sequencing in the FV promoter region was also used to investigate the presence of polymorphic candidates to increase plasma FV levels in 10 subjects with FV levels in the highest percentiles (90 and 100 percentiles, FV:C range: 183 to 287). We only detected a previously reported polymorphism (~426 G/A) (35) that was extensively genotyped. Heterozygotes (n=121) for this marker showed mean FV levels (127.7±42.5) indistinguishable from that found in the whole population (Table 2). Frequencies observed for all FV polymorphisms investigated were consistent with those predicted by the Hardy–Weinberg equilibrium.

Genotyping of Subjects Carrying FV Leiden Mutation

In the doubly heterozygous condition with FV Leiden, the Met2120Thr and Asp2194Gly changes could produce,
through a partial pseudohomozygous mechanism, a relative increase in plasma levels of APC resistant molecules. A significant (ANOVA, \(F = 4.44, P = 0.014\)) contribution to APCR of these polymorphisms was detectable in 122 unrelated thrombotic patients carriers of FV Leiden (Table 3). Seven were doubly heterozygous for the FV Leiden and the 2120Thr allele and showed a significant (\(P < 0.05\)) decrease in APC ratios as compared with simple FV Leiden carriers (\(n = 99\)). Although slightly decreased, APC ratios in the 16 doubly heterozygous for FV Leiden and the 2194Gly allele did not significantly differ from that of the 2194Asp and FV Leiden carriers.

Recombinant FV Expression

To corroborate the observations indicating positive association between markers and FV levels, we evaluated in vitro the functional impact of threonine at position 2120 of FV C2 domain. Expression in eucaryotic COS-7 cells of the recombinant 2120Thr-FV was compared with that of Wt–FV and 2194Gly–FV as control. Western blots of recombinant FV molecules in medium showed approximately similar amounts of proteins with indistinguishable migration pattern. In 10 independent transfections (Table 5), the cofactor activity of the secreted 2120Thr-FV measured by thrombin generation assay (see Methods) was approximately two thirds of Wt–FV (\(P = 0.043\)), which is a reduction (\(P < 0.001\)) comparable to that of 2194Gly–FV.

The levels of FV antigen (Table 5) in the conditioned media were higher for the Wt–FV than for the 2120Thr–FV (\(P = 0.006\)) or the 2194Gly–FV (\(P = 0.030\)). Significant mean differences in transfection efficiency were excluded by the luciferase assays.

### Table 3. APC Ratio in Thrombotic FV Leiden Carriers

<table>
<thead>
<tr>
<th>FV Genotypes</th>
<th>Subjects</th>
<th>APC Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden/Wt</td>
<td>99</td>
<td>81.2</td>
</tr>
<tr>
<td>FV Leiden/2120Thr</td>
<td>7</td>
<td>5.7</td>
</tr>
<tr>
<td>FV Leiden/HR2</td>
<td>16</td>
<td>13.1</td>
</tr>
<tr>
<td>All genotypes</td>
<td>122</td>
<td>100.0</td>
</tr>
</tbody>
</table>

FV haplotypes are separated by a slash. 
FV Leiden indicates haplotype 506Gln 1299His 2120Met 2194Asp; Wt, haplotype 506Arg 1299His 2120Met 2194Asp; 2120Thr, haplotype 506Arg 1299His 2120Thr 2194Asp; HR2, haplotype 506Arg 1299Arg 2120Met 2194Gly.

n and % indicate number and percentage of subjects, respectively.
APC ratio was reported as mean±SD.
ANOVA, \(F=4.44, P=0.014\)
*Significantly different from the Wt group (Bonferroni t test, \(P<0.05\))

### Table 4. FV Polymorphisms and Venous Thrombosis in 290 FV Leiden Carriers

<table>
<thead>
<tr>
<th>FV Polymorphisms</th>
<th>Symptomatic Subjects (n=145)</th>
<th>Asymptomatic Subjects (n=145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6533T/C (Met2120Thr)</td>
<td>TT</td>
<td>135</td>
</tr>
<tr>
<td>(x^2=2.899) ((P=0.089))</td>
<td>TC</td>
<td>10</td>
</tr>
<tr>
<td>6755A/G (Asp2194Gly)</td>
<td>AA</td>
<td>127</td>
</tr>
<tr>
<td>(x^2=6.800) ((P=0.009))</td>
<td>AG</td>
<td>18</td>
</tr>
</tbody>
</table>

n indicates number of subjects.

### Table 5. FV Levels in Medium of Recombinant FV

<table>
<thead>
<tr>
<th>FV Mutants</th>
<th>n</th>
<th>FV Activity</th>
<th>FV Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1299His, 2120Met, 2194Asp (Wt)</td>
<td>10</td>
<td>100* †</td>
<td>46.3±10.08§</td>
</tr>
<tr>
<td>1299His, 2120Thr, 2194Asp</td>
<td>10</td>
<td>66±33†</td>
<td>24.7±19.7‡</td>
</tr>
<tr>
<td>1299His, 2120Met, 2194Gly</td>
<td>10</td>
<td>66±24*</td>
<td>34.6±12.1§</td>
</tr>
</tbody>
</table>

n indicates number of independent transfections.
FV activity was expressed as % of Wt; FV antigen was expressed as ng/mL (mean±SD).
t-tests: *\(P<0.001\); †\(P=0.043\); §\(P=0.006\); ‡\(P=0.030\)
Overall, the expression of recombinant FV indicates a proportional decrease in activity and antigen for the 2120Thr-FV, similar to that of the 2194Gly-FV.

**Discussion**

To investigate FV gene variation contributing to FV levels in plasma, we have used a systematic approach based on an extensive investigation of a genetically stable microsatellite marker and several SNPs. Moreover, sequencing of the promoter region, and of a large portion of coding regions, was performed in selected subjects. We have combined genotyping in a relatively large cohort of subjects characterized for FV levels with expression of recombinant FV carrying candidate mutations. This approach provides us with valuable information to further investigate the association between clinical phenotypes and FV genotypes.

The population in this study was enrolled in a regional study of coronary heart disease and consequently is characterized by older age than subjects contributing to the normal pooled plasma who were selected among several age classes. The observation that FV levels increase with age could partially explain the high mean FV levels observed in our sample.

Several microsatellite genotype groups, characterized by the highest FV levels, ie, the first 6 groups listed in Table 1, showed very similar mean FV activity values, which does not suggest the presence of frequent underlying FV mutations leading to significant differences in FV expression. The possibility that promoter mutations genetically linked to frequent microsatellite alleles would have eluded detection was ruled out by nucleotide sequencing in the 5′ region, which was performed in subjects selected for FV levels. The sequencing approach, extended to 80% of coding regions, did not reveal any new gene variation, and genotyping for polymorphisms in the 5′ region (−426G/A) and in exon 16 (Met1736Val) did not show association with high FV levels. The secretion rate of the 1736Val FV has been previously reported for other coagulation parameters such as APC resistance. Decreased FV antigen levels were also found in media of cells expressing the recombinant FV mutants, which suggests the presence of mild quantitative defects. Decreased FV antigen levels were also found in plasma of carriers of both polymorphisms (D.G., unpublished results).

The Asp2194Gly substitution has previously been suggested as a functional candidate for the effect of the FVHR2 haplotype on FV levels, either by the study of a small group of FVHR2 homozygotes or by expression studies. The presence in our population study of 10 subjects carrying a rare, naturally occurring FVHR2 variant lacking the Asp2194Gly change provided strong evidence, based on an in vivo model, for the functional role of the 2194Gly FV variant in lowering FV levels. The Asp2194Gly substitution, among the several amino acid changes characterizing the FVHR2 haplotype, is the functional determinant of this phenotype.

These genetic markers of relatively reduced FV levels were present in approximately one fifth of the population and provide examples of frequent and subtle inherited components that could interact with major thrombophilic mutations. The Met2120Thr and Asp2194Gly polymorphisms, associated with a 25% reduction of FV levels, could produce, in the doubly heterozygous condition with FV Leiden, an approximately 2-fold relative excess of FV Leiden molecules in plasma, which is a partially pseudohomozygous condition. Accordingly, we found that the 2120Thr allele was associated with increased APC resistance in FV Leiden patients affected by venous thromboembolism.

The relationship between carrying these polymorphisms and thrombosis was also investigated in carriers of FV Leiden by comparing genotype distribution in VTE patients and in asymptomatic subjects. This analysis further supported a prothrombic role of the Asp2194Gly change and of the associated HR2 haplotype. The same approach failed to find significant differences for the less frequent Met2120Thr polymorphism, which, however, showed a distribution in patients and asymptomatic subjects similar to that observed for the 2194Gly allele.

These functional polymorphisms are appropriate markers for further association studies aimed at investigating the relationship of FV levels with thrombotic disease and, particularly, the gene–gene and gene–environment interactions producing the variable clinical expressivity of the FV Leiden.

**Acknowledgments**

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**References**


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