Perthes’ disease and the search for genetic associations

COLLAGEN MUTATIONS, GAUCHER’S DISEASE AND THROMBOPHILIA

The role of heritable thrombophilic risk factors in the pathogenesis of Perthes’ disease is controversial. The clinical and radiological findings of Perthes’ disease may be indistinguishable from those of Gaucher’s disease, and the most common Jewish N370S Gaucher mutation is threefold greater in patients with Perthes’ disease. Familial osteonecrosis of the femoral head is associated with variant mutations of collagen type II (COL2A1 mutations). We therefore studied the potential role of genetic thrombophilia and the Gaucher and COL2A1 mutations in children with Perthes’ disease.

Genomic DNA of 119 children with radiologically-confirmed Perthes’ disease diagnosed between 1986 and 2005 was analysed for the thrombophilic polymorphisms Factor V Leiden, 677T-MTHFR and FIIG20210A. The results were compared with those of a group of 276 children without Perthes’ disease. DNA was also analysed for the Gaucher mutations N370S, G insertion (84GG), L444P, Intron 2 (IVS2+1G>A) and R496H. Enzymic assays confirmed the Gaucher disease status. Collagen (COL2A1) mutations of the 12q13 gene were also analysed. The prevalence of thrombophilic markers was similar among the 119 patients with Perthes’ disease and the 276 control subjects. The prevalence of the Gaucher mutation was consistent with Israeli population carriership data and did not confirm an earlier-claimed association with Perthes’ disease. All 199 patients were negative for the studied COL2A1 mutations.

We found no genetic association between Perthes’ disease and either Gaucher’s disease or COL2A1 mutations or increased genetic thrombophilia among our patients compared with the control group. A systematic review of case-control studies suggested that there was a positive association between Perthes’ disease and Factor V Leiden. The impact of this association upon the disease, although not consistent across the studies, remains unclear.

In Perthes’ disease it has been suggested that disruption of the blood supply to the femoral head results in venous occlusion and hypertension and bone death. Intravascular thrombosis may be the causative mechanism, but the role of heritable thrombophilic risk factors is unproven.

Gaucher’s disease, the most prevalent lysosomal storage disorder, has vast phenotypic heterogeneity. One of its most debilitating features is avascular necrosis of large joints. It is an autosomal recessive disorder caused by more than 200 mutations of the β-glucocerebrosidase gene. The most frequent is the N370S (1226G) mutation which occurs in Ashkenazi Jews in whom there is a predilection for the non-neuronopathic type I with an incidence estimated to be 1:850. Clinical and radiological findings of avascular necrosis due to Perthes’ disease may be indistinguishable from those of Gaucher’s disease. In a previous study we found a threefold increase in the prevalence of the most common Jewish N370S Gaucher mutation in a selective series of patients with Perthes’ disease. The potential association of Perthes’ disease with other Gaucher mutations has not previously been studied. Familial osteonecrosis of the femoral head has recently been found to be associated with variant mutations of collagen type II. The possible impact of such mutations on the pathogenesis of Perthes’ disease has not previously been tested. Since Perthes’ disease is a condition of multifactorial origin and may be attributed to genetic as well as environmental risk factors, our aim was to evaluate the potential role of all these genetic factors in a cohort of patients with Perthes’ disease. We also made an exhaustive search of the literature in order to carry out a meta-analysis of the associations of Perthes’ disease with genetic thrombophilia.
Patients and Methods
We were able to obtain all the records and recall 119 of 162 patients with Perthes’ disease who had been treated at our department between 1986 and 2005. All had parental consent and were willing to undergo all the required blood tests and DNA sampling. The diagnosis of Perthes’ disease had been made by clinical findings and radiological assessment. There were 91 males (76.5%) and 28 females (23.5%) with a mean age at diagnosis of six years (1.0 to 14.9). Their ethnic backgrounds were Ashkenazi Jewish (37 patients; 31.1%), Sephardic Jewish (50 patients; 42.0%), Jewish of mixed Ashkenazi and Sephardic origin (25 patients; 21.0%) and Arabs (7 patients; 5.9%). Most of the patients (92; 77.3%) lived in urban areas and the remainder in rural areas. The records were reviewed for the age of onset and extent of the disease, with severity being graded according to the Catterall classification for 115 (96.6%) patients, and according to the modified lateral pillar classification for 52 patients (43.7%). A detailed family history of Perthes’ disease, Gaucher’s disease or hypercoagulability was obtained from their parents. The study was approved by the institutional review board.

For assessment of thrombophilic DNA polymorphism frequency in our population, we recruited 276 children from paediatric surgical and haematology departments. We also obtained cord blood samples of neonates from one large medical centre. Inclusion criteria for the control group were either term neonates or children admitted for elective surgery, trauma or elective evaluation. Patients with sepsis, acute febrile illness, coagulation abnormalities, active cancer or avascular consent for blood sampling was obtained from all the children’s guardians.

For the DNA preparation blood was collected into 3.8% (0.109 M) trisodium citrate anticoagulant in a 9:1 ratio (blood citrate). The citrated blood was centrifuged within 30 minutes at 2000 g (20 minutes) and plasma aliquots were stored at -35°C. Since thrombophilic risk factors such as the levels of protein C, the presence of antiphospholipid antibodies etc., may vary with age or be affected by acute phase reactions, and since most of the children could be sampled only once with no option for confirmatory testing of the subject or other family members, we assessed thrombophilia in our study and control groups solely by the frequency of thrombophilic DNA polymorphisms.

Genomic DNA was extracted from EDTA-anticoagulated blood samples using standard methods. Factor V Leiden (FVL) was detected by polymerase chain reaction (PCR) amplification of a 267 bp fragment and MNII digestion, as previously described. The C677T polymorphism in the methylene-tetrahydrofolate reductase (MTHFR) gene was identified using the Hindl cleavage of a 198 bp PCR-amplified product. We used a previously described modification of the technique used by Poort et al to identify the G20210A substitution in the factor II gene (FIIG20210A). DNA for the detection of Gaucher mutations was extracted from blood spots on capture cards (Genta systems, Minneapolis, Minnesota) according to the manufacturer’s instructions. The following Gaucher mutations were analysed: N370S, G insertion (84GG), L444P, Intron 2(IVS2+1G>A) and R496H. Enzymic assays for β-glucosidase and chitotriosidase activity were performed for confirmation of the status of Gaucher’s disease according to standard techniques. Collagen (COL2A1) mutations of the 12q13 gene were analysed as previously described.

Statistical analysis. This was performed using SPSS software version 14 (SPSS Inc., Chicago, Illinois). Relationships among categorical variables were assessed using the chi-squared test, Fisher’s exact test and logistic regression. Follow-up time was characterised by the median and was compared across groups by the Wilcoxon test. The Mantel-Haenszel test was used to compare the results of different studies. The Breslow-Day test was used to calculate the homogeneity of different studies. A p-value ≤ 0.05 was considered to be significant.

Results
Femoral involvement was on the left side in 57 patients (47.9%), on the right in 45 (37.8%) and was bilateral in 17 (14.3%). The Catterall classification of 115 patients showed that five patients (4.3%) had group-1 disease, 11 (9.6%) group-2, 33 (28.7%) group-3 and 66 (57.4%) group-4 (severe) disease. Of the 52 patients who had been classified by Herring’s modified lateral pillar classification, three (5.8%) had group-A disease, 21 (40.4%) group-B, eight (15.4%) group B-C disease and 20 (38.5%) group-C disease.

The parents of all the patients were healthy. The family history was negative for Gaucher’s disease in all but one, and negative for early age (< 50 years) of thromboembolic events. Only four patients had familial Perthes’ disease of whom two were siblings in the current study.

The prevalence of thrombophilic markers was similar among the patients and the control group (Table I). A total of 91 patients (76.5%) had no genetic thrombophilia, six (5.0%) were heterozygous for FVL alone, four (3.4%) were heterozygous for FIIG20210A alone, 17 (14.3%) were homozygous for 677T MTHFR alone and one patient (0.8%) had combined thrombophilia, being both FVL heterozygous and homozygous for 677T MTHFR.

No relationship was found between the presence of thrombophilia and bilateral versus unilateral presentation of the Catterall classification at diagnosis. There was a higher percentage of Catterall groups III and IV among the children with thrombophilia (17 of 18, 94.4%) than among those without thrombophilia (47 of 57, 82.5%), but this difference was not significant (Fisher’s test, p = 0.28). The median follow-up times were 3.0 and 2.75 years for those with and without thrombophilia, respectively. The follow-up time distributions did not differ significantly (Wilcoxon test, p = 0.44).

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With regard to Gaucher mutations, four patients were heterozygous and one patient homozygous for the N370S mutation. Thus, the prevalence of the N370S mutation among our patients was 2.5% (6 of 238 alleles). No cases were positive for the other mutations. Of the 55 patients who had been screened for β-glucosidase enzymic activity, 26 had a low threshold (< 1.0). These findings were lower than the Ashkenazi Israeli population carriership data of 5.8%,12 and did not coincide with the association with Perthes’ disease found in a smaller preliminary study.13

All the patients were negative for the studied COL2A1 mutations.

Discussion
The incidence of Perthes’ disease varies greatly between different regions and populations throughout the world. It is more common in Caucasians, with a reported annual incidence ranging between 5.1 and 15.6 per 100 000.23-27 In addition, the incidence is associated with demographic factors, such as population density and socio-economic status.28-30 Environmental risk factors, such as smoking31 and diet (low serum manganese levels), have also been suggested.32 There may also be a genetic predisposition for Perthes’ disease. There are differences between genders in that boys are affected eight times more often than girls, and between different ethnic groups with a higher prevalence in Caucasians relative to Asians and Blacks, in addition to reported familial clustering.24,25,33,34

Our study was carried out on a selected cohort of patients with Perthes’ disease (3:1 male predominance, 86.1% Catterall groups II to IV) referred for evaluation in our tertiary referral centre. Their data were compared with those of a general Israeli paediatric population. Despite the referral bias of studying mainly severely affected patients, there were no genetic associations with thrombophilia, Gaucher’s disease or familial osteonecrosis. The last is associated with collagen type-II mutations,14 but unlike Perthes’ disease, it is not affected by environmental risk factors. The lack of any common aetiology with Perthes’ disease was confirmed by the absence of collagen type-II mutations. Our earlier study showed an association of the N370S Gaucher mutation with the risk of Perthes’ disease.13 This finding was not confirmed when re-tested in the current larger case-controlled study group using a larger scale of Gaucher mutations. This is probably due to selection bias. We believe that Perthes’ disease may be misdiagnosed in patients of Ashkenazi origin who present with bone manifestations indicative of Gaucher’s disease, even although a genetic association between both diseases has not been established.

Since the first reports by Glueck et al14,35,36 a total of 180 patients with Perthes’ disease who were tested for hypercoagulability have been reported in case reports and small series.5,9,37-39 In those uncontrolled studies, few patients tested positive for thrombophilia (not including FVL). Three of the 180 children were reported to have mildly low antithrombin levels, five had low protein C activity and three had low protein S antigen. No confirmatory levels for these thrombophilic factors were obtained from family members and data on the vitamin-K status were not available. Interestingly, 11 of the 180 patients with Perthes’ disease (6.1%) were FVL heterozygous. One with recurrent Perthes’ disease showed no thrombophilia.

The results of 11 case-control studies which evaluated thrombophilia in Perthes’ disease are summarised in Table II. This table does not include some published findings. First, the study by Glueck et al15 considered that protein C levels of < 65% and protein S levels < 76% were pathological, but these levels may increase with age and adjustments need to be made accordingly. No confirmatory tests were performed either in that study or in an earlier one by the same group.36 We have therefore included in Table II only the activated protein C resistance and FVL results of the first study.4 Studies referring to non-classical thrombophilic tests such as provoked fibrinolysis4 and tissue factor fibrinolysis inhibitor,40 have also been excluded.

Secondly, among plasma assays obtained for thrombophilic risk factors, only one reportedly found the value of the partial thromboplastin time to be prolonged in patients compared with a control group.41 The study, however, did not test for lupus anticoagulant, a potential cause of thrombophilia as well as for a prolonged partial thromboplastin time.

Thirdly, two studies5,42 evaluated antiphospholipid antibodies, but only one showed a significantly higher prevalence in patients compared with a control group.42 However, the definition of antiphospholipid antibodies requires confirmatory antibody testing on at least two occasions 12 weeks apart in order to avoid false-positive results due to acute phase reactions43 such as after vaccinations or infections etc. We were therefore unable to refer to the antiphospholipid antibodies results of this study.

Lastly, markers of clot lysis such as platelet activation, thrombin-antithrombin complexes, di-mers, homocysteine, lipoprotein (a) or genetic plasminogen activator inhibitor polymorphism were only rarely tested by some

<table>
<thead>
<tr>
<th>Marker</th>
<th>Perthes’ disease (%)</th>
<th>Control group (%)</th>
<th>p-value</th>
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<tr>
<td>Factor V Leiden</td>
<td>7 (5.9)</td>
<td>13 (4.7)</td>
<td>0.81</td>
</tr>
<tr>
<td>FII(G20210A)</td>
<td>4 (3.4)</td>
<td>11 (4.0)</td>
<td>0.99</td>
</tr>
<tr>
<td>MTHFR677T</td>
<td>18 (15.1)</td>
<td>41 (14.9)</td>
<td>0.93</td>
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investigators.\textsuperscript{9,42,44} They did not show any significant differences between patient and control groups. Overall, the case-controlled trials have tested a total of 478 patients and 953 controls. No significant differences between patients and controls were found in baseline coagulation tests, antithrombin activity, protein C activity, free protein S antigen levels and antiphospholid antibodies.\textsuperscript{4,6-8,38,41,44-48}

In the systematic review of the literature summarised in Table II, it was shown that the prevalence of thrombophilic polymorphisms 677T-MTHFR and FII G20210A were equally distributed among the patients and control groups. Of a total of 475 patients tested for FVL in the cited studies, 31 were heterozygous and five were homozygous for FVL (36 patients; 7.5%) compared with 23 heterozygous FVL cases of 953 tested controls (2.4%) (Mantel-Haenszel test; \( p = 0.00029 \)). With regard to the antiphospholid antibodies, lower than normal values which may stem from either FVL or acquired conditions such as acute-phase elevation of Factor VIII, were demonstrated in 40 of 344 patients (11.6%) tested, compared with 19 of 509 (3.7%) control subjects. This difference was statistically significant (Mantel-Haenszel test, \( p < 0.001 \)). However, the relationship between Perthes’ disease and FVL was not consistent across studies for the homogeneity of the odds ratio (Breslow-Day test; \( p = 0.0477 \)). Despite the limitations of such calculations we feel that due to the rarity of Perthes’ disease and the paucity of well-conducted case-controlled studies regarding thrombophilia in such patients, a summary of the ratios of genetically-tested thrombophilic risk factors such as FVL, MTHFR and FII G20210A certainly sheds more light upon the issue. Nevertheless, the large variability noted among the studies prevents performance of a valid meta-analysis and warrants further research.

In conclusion, although most recent case-control studies testing thrombophilia in patients with Perthes’ disease failed to show positive correlations of thrombophilic risk factors and the occurrence of the disease, our review of the literature demonstrated that a genetic association of FVL and Perthes’ disease could not be ruled out. Further prospective large multicentre studies are required to determine the potential role of this association in cases of severe familial disease.

### Supplementary material

A further opinion by Mr A. Catterall is available with the electronic version of this article on our website at [www.jbjs.org.uk](http://www.jbjs.org.uk)

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References