Full Title: Fibrinogen $\alpha$Thr312Ala polymorphism is associated with Chronic Thromboembolic Pulmonary Hypertension

Short title: Fibrinogen $\alpha$Thr312Ala in CTEPH

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Abstract

Rationale

Although Chronic Thromboembolic Pulmonary Hypertension (CTEPH) is characterised by the persistence of organised thrombus, few prothrombotic risk factors have been identified in subjects with the disease.

Objectives

This study compares the prevalence of eight functionally relevant haemostatic polymorphisms between CTEPH subjects and healthy controls.

Methods

Genomic DNA was isolated from 214 CTEPH and 200 healthy controls and analysed for Factor V Leiden, Prothrombin G20210A, PAI-1 4G/5G, tPA C7351T, Factor XIII G100T, Fibrinogen AαThr312Ala, Fibrinogen BβArg448Lys and Fibrinogen BβG455A polymorphisms.

Results

A significant difference was demonstrated in Fibrinogen AαThr312Ala genotype (p=0.03) and allele (p=0.01) frequencies between CTEPH subjects and controls. Presence of the Ala allele significantly increased the risk of CTEPH (OR 1.68; p=0.01 95%CI 1.13, 2.49).

Conclusions

The Fibrinogen Aα312 Ala allele alters fibrinogen α–α chain cross-linkage and has previously been associated with both an increased risk of embolisation and an
increased resistance to thrombolysis. An association between this polymorphism and CTEPH therefore supports an embolic aetiology for this disease, and may provide a mechanism by which thrombus persists following an acute event.

Abstract word count: 181 words

Keywords: coagulation, fibrinolysis, polymorphisms, thromboembolic, pulmonary hypertension
Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) is increasingly recognised as an important cause of pulmonary hypertension, and is generally considered to represent an uncommon consequence of acute pulmonary embolic (PE) disease(1). CTEPH has been reported to occur after approximately 4% of cases of acute PE(2), whilst retrospective studies have described a preceding DVT in approximately one third of cases(3, 4). It is therefore presumed that following an acute embolic event, thrombus persists and organises, resulting in chronic obstruction to pulmonary arterial blood flow.

Given the key role of thrombus formation in CTEPH, one would intuitively expect there to be some aberration of haemostasis, at either a local or systemic level. However, although hereditary thrombophilic conditions are commonly associated with acute PE and DVT(5, 6), no such conditions have been strongly linked with CTEPH(7). Elevated serum levels of antiphospholipids and factor VIII have been described(8, 9), but it is unclear whether these represent primary or secondary phenomena. As a result no prothrombotic pathogenic mechanisms have been clearly shown to predispose to CTEPH. It is possible however that other steps in the haemostatic process, such as fibrin stabilisation or fibrinolysis, may be involved in the pathophysiology of the disease(10). A number of single nucleotide polymorphisms (SNPs) have been described that predispose to arterial and venous thrombotic disease, by affecting these more global aspects of haemostasis(11-20). This case control study therefore uses a candidate gene approach to determine the potential relevance of these SNPs in CTEPH.
Methods

Subjects
All consecutive Caucasian patients attending Papworth Hospital with a diagnosis of CTEPH between 1999 and 2006 were offered entry to the study. CTEPH was diagnosed through clinical evaluation, right heart catheterisation and appropriate imaging (chest radiography, VQ scintigraphy, CT pulmonary angiography, MR pulmonary angiography and catheter directed angiography) as per a standardised protocol, described elsewhere(4, 21). CTEPH subjects (n=214) included those with proximal (n=169) disease and distal (n=45) disease. 200 healthy controls (100 male and 100 female) were recruited from a National Blood Service site in West London and comprised Caucasians aged between 18 and 65 years.

The study protocol was approved by the local ethics committee. Written informed consent was obtained from each subject before entry to the study.

Genotyping
Genomic DNA was extracted from whole blood (Nucleon extraction kit, Amersham Biosciences) and analysed for single nucleotide polymorphisms (SNP) using commercially available primers (Taqman® SNP genotyping assay, Applied Biosystems) on an ABI Prism®7900HT Sequence Detection system. The eight SNPs studied were common genomic variations of the following haemostatic factors; Factor V (Factor V Leiden mutation or G1691A; rs6025)(11), Prothrombin (G20210A; rs 1799963)(11), PAI-1 (4G/5G; rs1799768)(11), tPA (C7351T; rs2020918)(17),
Factor XIII (G100T; rs5985)(15), Fibrinogen Aα (Thr312Ala, rs6050) and Fibrinogen Bβ (Arg448Lys, rs4220 and G455A, rs1800790)(22).

Statistics

Genotype and allele frequencies were calculated for each locus. Observed frequencies in controls were compared with those predicted by the Hardy-Weinberg equilibrium equation using the chi-square test. Linkage disequilibrium (LD) between SNPs was calculated from maximum likelihood haplotype frequencies estimated by the Expectation Maximization (EM) algorithm(23).

Genotype and allele frequencies were compared between CTEPH and control subjects using the chi square test, or the Fisher’s exact test in the case of low counts. The risk of disease associated with each genotype (genotype model) was estimated by logistic regression analysis where SNP genotype was coded in a three-level fashion ie rarest homozygous genotype, heterozygous genotype and most common homozygous genotype, with the latter being the reference category. For SNPs where there were no rare homozygous subjects, genotype was coded as heterozygous and compared to the most common homozygous genotype. The nature of the relationship between each SNP and risk of disease was also examined using models of dominant and recessive inheritance (allele model). In the dominant case, having one or more copies of the rare allele was compared to having no copies. In the recessive case, having two copies of the rare allele was compared to having one or no copies. Multiple logistic regression models of the effect of pairs of SNPs were used to assess whether there was any synergistic effect of having two
SNPs. In view of gender differences between the two groups, all analyses were also repeated using multiple logistic regression models that adjusted for gender. Adjustment for multiple tests was not made for single SNP analyses as each has previously been shown to be independently associated with an increased risk of thrombosis and this study aimed to re-address these hypotheses in the context of CTEPH. As such, calculations for each SNP constituted a separate and distinct analysis.

All statistical analyses were performed using SPSS v13 (SPSS Inc, Chicago, USA) software.

Results

Subjects and samples

Baseline demographic characteristics of the CTEPH subjects are shown in Table 1. Only gender information was available for the 200 healthy controls, although it is known that they were Caucasian, aged 18-65 years and fulfilled the criteria required for blood donation.

The mean yield from SNP analyses was 96.3%. All SNPs were in Hardy-Weinberg equilibrium, except for Fibrinogen Bβ448 (p=0.045). Only Fibrinogen Bβ448 and Fibrinogen Bβ455 were demonstrated to be in LD ($r^2=0.91$).

The genotype and allele frequencies for each of the SNPs in the two groups are shown in Table 2.
Polymorphism frequencies

A significant difference was demonstrated in the Fibrinogen AαThr312Ala genotype (p=0.03) and allele (p=0.01) frequencies between the CTEPH and control groups (Table 2). The three-level genotype model showed an association between the Fibrinogen AαThr312Ala SNP and CTEPH, though this did not reach significance for homozygotes (OR 1.65 p=0.02 95%Cl 1.13, 2.49 for heterozygotes and OR 1.82 p=0.09 95%Cl 0.92, 3.61 for homozygotes). A dominant model of inheritance of the Ala allele (ie one or two copies of the Ala allele compared to none) showed increased odds of CTEPH (OR 1.68; p=0.01 95%Cl 1.13, 2.49). There was no evidence of confounding of the relationship between the Fibrinogen AαThr312Ala SNP and CTEPH by gender (dominant model: females vs males OR 1.06; p=0.77 95%CI 0.72, 1.57; three-level genotype model: females vs males OR 1.06; p=0.78 95%CI 0.72, 1.57)

There was a difference was in the Factor V Leiden genotype frequencies between the two groups, although this did not reach statistical significance (p=0.051). The Factor V Leiden mutation was associated with an increased risk of CTEPH (genotype model, no rare homozygotes: OR 2.54; p=0.06 95%Cl 0.97, 6.68), although again this was not statistically significant.

There were no other significant differences between the two groups and no evidence of synergism between SNP pairs.
The six SNPs that failed to show an association with CTEPH were also used as a form of genomic controls, to test for population stratification. The Kolmogorov-Smirnov goodness-of-fit test of the chi-square statistics for the association between each SNP and disease demonstrated that these followed a distribution that was not significantly different from a chi-square distribution (p=0.35). These results suggest a lack of stratification.

**Discussion**

This study demonstrates that the presence of the Ala allele at position 312 of the fibrinogen \(\alpha\) chain is associated with an increased odds of being diagnosed with CTEPH. In addition, a significant association with the Factor V Leiden mutation was also noted. These are the first haemostatic polymorphisms to be reported in a large series of CTEPH subjects, and thus may add to our understanding of the pathophysiological mechanisms behind this enigmatic disease.

Although CTEPH is widely considered to be a consequence of acute pulmonary embolic disease(1, 24), the evidence to support this belief is largely circumstantial. One concern that has fuelled the debate over the aetiology of this disease is the discrepancy that exists between the thrombophilic profiles of DVT patients compared with CTEPH patients(25). However, rather than viewing this as a barrier to the ‘embolic theory’ of CTEPH, it is worth considering what may distinguish the minority of patients who develop CTEPH following a simple DVT, from the vast majority that do not. In this context, it is not just important to consider the haemostatic factors that
predispose to thrombus formation, but to also consider those that determine how thrombus is subsequently handled. A key factor in this process is clearly the fibrinogen molecule, which provides the essential building blocks for forming fibrin clot. Abnormalities within this molecule may not only promote thrombus formation, but also delay its degradation, thus encouraging the switch from thrombus resolution to thrombus organisation.

The fibrinogen molecule comprises three pairs of polypeptide chains (Aα, Bβ and γ) that are linked by disulfide bonds and arranged into three regions; a central (E) region, containing fibrinopeptides A and B and the amino acid termini of all six chains, and two distal (D) regions, each formed by the unpaired carboxyl termini of the Aα, Bβ and γ chains(26). D regions also include a globular Cα domain, formed by the terminal two thirds of the α chain. Within a given fibrinogen molecule, the two Cα domains are tethered together in close proximity to the E region(27). Following activation of the coagulation cascade, thrombin cleaves fibrinopeptides A and B from fibrinogen, producing fibrin monomers and initiating clot formation. Thrombin simultaneously activates factor XIII, promoting cross linkage between newly-formed fibrin oligomers. As part of this process, Cα domains, now untethered from the E region following cleavage of fibrinopeptide B, are encouraged to bind with Cα domains from neighbouring fibrin molecules(28). The resulting α-α linkage strengthens the association between adjacent protofibrils and stabilises the developing fibrin structure. This allows formation of a fibrin structure that is stronger, more rigid and more resistant to thrombolysis(29).
The pivotal role of the Cα domain in fibrin polymerisation has been highlighted previously by an in vitro study which demonstrated that monoclonal antibodies directed against this domain inhibit the formation of fibrin polymers from monomers(30). Recombinant fibrinogen lacking the Cα domain has also been shown to form clot in vitro that is less stiff and more susceptible to thrombolysis than clot formed in the presence of normal fibrinogen(31). Further information about the role of the Cα domain has also been gained from studying hereditary dysfibrinogenaemias(32). Dusart syndrome, caused by an AαArg554Cys substitution, is associated with increased α−α chain linkage, and hence enhanced lateral aggregation of fibrin protofibrils. Fibrin clots from patients with the disease tend to be stiff, impermeable and resistant to thrombolysis in vitro(33, 34). Clinically, patients suffer recurrent thrombotic complications, and are particularly prone to embolic phenomena(35). Conversely, the dysfibrinogen Caracas II, caused by an AαSer434Asn substitution, is associated with reduced α−α chain linkage compared with normal fibrinogen. Clots are characteristically loosely packed in structure, and have normal stiffness but increased permeability(36). Patients with the disorder are typically asymptomatic and do not suffer any haematological complications(36).

AαThr312Ala is a well recognised polymorphism of the Aα gene that codes for a threonine-to-alanine substitution at residue 312 of the Cα domain(37). Although the functional relevance of this polymorphism is still unclear, the area immediately surrounding this site is known to be central to both factor XIII activation and to factor XIII-dependent α chain cross-linking and α2-antiplasmin binding(38, 39). Clinically, homozygosity for the Ala allele has been associated with acute pulmonary embolic disease but not DVT(14). The presence of the Ala allele, in both heterozygotes and
homozygotes, has also been associated with higher post-stroke mortality in subjects in atrial fibrillation but not sinus rhythm(13). It has therefore been suggested that the Ala substitution may predispose to embolic phenomena, through the formation of more ‘brittle’ clot(40). In the case of DVT, such clot would be more likely to fragment and dislodge as it propagates proximally and comes under the influence of oblique flow from conjoining veins. The results of this present study support this hypothesis, and contribute further to the evidence that CTEPH has an embolic aetiology.

Evidence from in vitro studies suggests that the AαThr312Ala polymorphism may have an additional pathophysiological role in CTEPH, other than simply predisposing patients to embolic phenomena. Clot formed from Ala-Ala subjects demonstrates more extensive α–α chain cross linking, increased fibre thickness and greater clot stiffness than is seen in Thr-Thr controls(40). Such clot also shows less permeability to flow, which in turn allows less access to fibrinolytic factors in vivo(41). These properties suggest that the Ala substitution enhances lateral aggregation of fibrin protofibrils and encourages formation of a more tightly packed fibrin structure. Although not specifically measured in these studies, such parameters are typically associated with clot that is more resistant to thrombolysis. Moreover, recent data suggests that fibrin derived from CTEPH patients is slower to lyse than fibrin derived from controls(10). This combined evidence implies that delayed fibrin degradation, possibly mediated in some by the AαThr312Ala polymorphism, is a pivotal step in the development of organised scar tissue from acute pulmonary embolic material in a subset of patients with CTEPH.
Our study also suggested a difference in the frequency of the Factor V Leiden mutation between CTEPH subjects and controls, although this difference did not reach statistical significance (p=0.051). The Factor V Leiden mutation, characterised by an Arg506Glu substitution, is resistant to cleavage by activated protein C (APC), and is therefore inactivated more slowly(42). Additionally, presence of this mutation interferes with the cofactor activity of factor V on APC-catalyzed inactivation of factor VIIIa(43). Both mechanisms promote a hypercoagulable state that has been significantly associated with the risk of developing DVT(44). Previous small studies have shown that the prevalence of this mutation in CTEPH is low, and not dissimilar to that of controls(9, 45). This present study examines a much larger cohort of patients and suggests a possible association, although the prevalence remains much lower than that found in patients with DVT. Given this data, a role for factor V Leiden in CTEPH cannot be fully discounted, particularly as factor VIII, a substrate for factor V, has already been implicated in the disease(8).

Although this study represents the largest of its kind in CTEPH, it does have several limitations. The use of ‘convenience’ controls that were not necessarily drawn from the same population as CTEPH cases may have confounded the study, although the results of the Kolmogorov-Smirnov test suggest that population stratification was not an issue. Furthermore, whilst there is evidence to support a pathophysiological role for both Fibrinogen AαThr312Ala and Factor V Leiden mutations in thrombotic disease, it is possible that these mutations themselves are not causal but instead are in linkage disequilibrium with other causal loci. Finally, the relatively small numbers of subjects involved meant that the study may not have been powered sufficiently to detect differences in SNP frequencies between the disease and control groups.
Differences that were found could have been chance findings in the group of individuals studied, and thus further studies are needed to confirm these results. If these future studies included a second control group of subjects with a history of completely resolved acute pulmonary embolism, a better understanding of the mechanisms underlying CTEPH could potentially be gained.

In conclusion, this study demonstrates an association between both fibrinogen A<sub>α</sub>Thr312Ala and Factor V Leiden mutations and CTEPH that adds to our understanding of the disease. Presence of these polymorphisms may not only predispose patients to embolic phenomena, but may also confer resistance to fibrinolysis that subsequently sets the stage for thrombus organisation.

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**Funding sources**

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**Conflict of interest statement**

None of the authors have any competing interests that relate to the contents of this manuscript.
References


Table 1: Baseline demographics of CTEPH subjects

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<thead>
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<th>CTEPH</th>
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<td></td>
<td>(n=214)</td>
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<tr>
<td>Age (years)</td>
<td>54.6 (17.7)</td>
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<tr>
<td>Gender (%male)</td>
<td>50.4</td>
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<tr>
<td>Right atrial pressure (mmHg)</td>
<td>9.3 (6.2)</td>
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<tr>
<td>mean Pulmonary Artery Pressure (mmHg)</td>
<td>46.4 (11.4)</td>
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<tr>
<td>Cardiac Index (l/min/m²)</td>
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<td>Total Pulmonary Resistance (WU)</td>
<td>12.8 (5.2)</td>
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</table>

Data presented as mean (standard deviation).
Table 2: Genotype and allele frequencies for the 8 candidate genes

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<tr>
<th>Genotype</th>
<th>CTEPH, n=214</th>
<th>Controls, n=200</th>
<th>p value</th>
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<td>Prothrombin (G20210) rs1799963</td>
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<td>G/A, n (%)</td>
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<td>198 (95.7)</td>
<td>196 (98.0)</td>
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<td>9 (2.2)</td>
<td>4 (1.0)</td>
<td>0.18</td>
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<tr>
<td>G, n (%)</td>
<td>405 (97.8)</td>
<td>396 (99.0)</td>
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<td>G, n (%)</td>
<td>397 (96.4)</td>
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<td>PAI-1 (4G/5G) rs1799768</td>
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<td>38 (19.2)</td>
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<td>99 (49.3)</td>
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<td>5G, n (%)</td>
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<td>tPA C7351T rs2020918</td>
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<td>22 (10.7)</td>
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<td>T, n (%)</td>
<td>133 (32.3)</td>
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<td>300 (76.1)</td>
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<td>Fibrinogen Aα312Thr/Ala rs6050</td>
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<td>76 (38.0)</td>
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<td>83 (40.7)</td>
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<td>71 (35.5)</td>
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<td>68 (16.3)</td>
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