PNH Screening in Patients with Recurrence of Thrombosis during Anticoagulant Therapy

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Introduction

Scientific background

Genetic basis of paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disorder of hematopoietic stem cells determined by somatic mutations in the *phoshpatidylinositol glycan-complementation class A* (PIG-A) gene. Such mutations cause an early block in the synthesis of the *glycosylphosphatidylinositol* (GPI) anchor, by which various proteins are attached to the cell membrane (Takeda et al, 1993). The subsequent partial (type II) or complete (type III) deficiency of GPI-anchored proteins with complement inhibition activity CD55 and CD59 renders PNH red blood cells abnormally sensitive to complement lysis (Parker et al, 2005). The consequence is a condition of persistent complement-induced intravascular hemolysis with brisk exacerbation that constitutes the primary clinical manifestation of the disease.

PNH and thrombophilia

Prof Luzzatto defines PNH as "the most vicious acquired thrombophilic state known in medicine " (Luzzatto et al, 2011).

Complement-induced intravascular hemolysis has been shown to contribute to the increased susceptibility to thromboembolism (TE) observed in patients with PNH (Hillmen et al, 2007). In particular, patients with larger PNH clones have a larger incidence of TE, and thromboembolic events have been temporally associated with increased hemolysis (Gralnick et al, 1995; Hall et al 2003). However, also patients with small, non-hemolytic clones, have been associated with an increased risk of thrombosis (Hall et al, 2003; Audebert et al, 2005).

The exact mechanism of this phenomenon is still to be understood: hemolysis is likely implicated in initiation of platelet activation and aggregation (Rother et al, 2005) and it was also suggested that complement may directly activate platelets as well as endothelial cells from PNH patients (Weitz et al, 2012). A state of the art analysis of the interconnections between complement disregulation and thrombophilia in PNH has been published recently (Hill, 2013)

The clinical counterpart of such phenomenon is that thrombosis is the first single cause of mortality in PNH, accounting for 40-67% of deaths (Hillmen, 2007). The increased incidence of thromboembolic events observed in PHN patients has been reported extensively: a retrospective study of 460 PNH patients (Peffault de Latour et al, 2008) showed that the 10-years cumulative incidence rate of TE events was 30.7 (95% CI 25.4-35.9), with an incidence at the time of diagnosis of 7.2%. Development of thrombosis was strongly related to poor survival, with 44% of the observed deaths related to TE (HR 15.4 [95% CI 9.3-25.4], P<0.001). A similar retrospective study on 220 PNH patients followed over a period of 46 years, showed (Socié et al, 1996) showed a relative risk of developing thrombosis of 10.2 (95% CI 6 –17). The risk of stroke in patients with PNH is more than 5 times greater than that of the general population, and is 8 times higher in patients <54 years of age (Hillmen, 2007; Gostynski M et al, 2006)

The cumulative analysis of 195 PNH patients recruited for three independent parent clinical studies (phase II and III) and the common open-label 102 weeks extension study

(Hillmen et al, 2007) showed that 18.5% of the TE events affected the mesentheric/splenic veins, 16.9% the hepatic/portal veins, 5.6% the cerebral sinuses. Only 15% of the thrombotic events were arterial, while in other cohorts of patients the cumulative incidence of arterial thrombosis was 39% (Lee et al, 2013). PNH patients have also been shown to be at high risk of developing Budd-Chiari syndrome, a condition otherwise observed only rarely, while 10.5 – 19.5% of patients with Budd-Chiari, has a PNH clone (Garcia Pagan, 2008, Darwish, 2009). Abnormalities suggestive of previous subclinical thrombosis were identified in 6 of 10 hemolytic PNH patients by high-resolution MRI imaging of the lung, cardiac and abdominal sectors, including patients on primary prophylaxis with warfarin (Hill et al, 2006).
40% of PNH patients present thrombosis at diagnosis (Hillmen, 2007) and 21% of patients had a clinical history of thrombosis at the time of PNH diagnosis (Lee, 2013).

Therapy of thrombosis in PNH
The consequence of the peculiar pathogenesis of thrombosis in PNH, is the scarce efficacy of standard anticoagulation therapy: the thrombotic events rate per 100 pt/yrs in PNH patients receiving anticoagulation prophylaxis was 11.54 (Hillmen, 2007). Similar data have been observed also in other cohorts of patients (Audebert et al, 2005) and is associated with an increased risk of bleeding (Palareti et al, 2007), including fatal haemorrhage (Moyo et al, 2004).
Eculizumab is a humanized monoclonal antibody that prevents complement activation by binding complement factor C5 (Thomas TC et al, 1996). In a clinical study (Hillmen et al, 2007) treatment with eculizumab strongly reduced the incidence of TE events in all PNH patients (from 39 events per patient/year to 3) as well as in patients receiving previous anti-thrombotics (from 10.61 to 0.62 events per patient/year)..

Research Rationale
In the light of the above considerations, we hypothesize that some of the otherwise unexplained occurrences/recurrences of thrombosis in patients undergoing primary or secondary prophylaxis with anticoagulant drugs may be due to undiagnosed PNH.
The therefore propose to screen for the presence of a PNH clone a cohort of patients presenting an episode of venous or arterial thrombosis during anticoagulation.
Patients with a recurrence of venous or arterial thrombosis during anticoagulation therapy, or with a first episode of venous or arterial thrombosis occurred during anticoagulation therapy not more than 5 years before screening, will be included in the study.

Objectives
To preliminarily determine the incidence of PNH clones in patients presenting a first episode/recurrence of venous or arterial thrombosis during anticoagulation therapy.

Study design
Multicentric, prospective and retrospective, non-interventional, exploratory study

Number of participating centres
10 (12), from Lombardia region, + 1 centralized laboratory

Number of patients
100

Length of the study
12 months

Number of subjects and sample size considerations
The total number of patients was determined on the basis of the exploratory nature of the study, the predicted recruitment rate and the rarity of the disease under investigation. A review of the literature suggests that the chosen numerosity will be sufficient to fulfil the endpoints of the study.

Criteria for diagnosis and enrolment

Inclusion criteria*:*
i. male or female subjects, age 18 or older
ii. capability to understand nature and implications of the study
iii. written informed consent
iv. Objective diagnosis of deep vein thrombosis or arterial thrombosis confirmed by instrumental tests, occurring during anticoagulation primary or secondary prophylaxis, at time of recruitment or not more than 5 years before.

Exclusion criteria
i. previous diagnosis of PNH
ii. one or more of the following conditions: active solid or haematologic cancer; liver cirrhosis; pregnancy or puerperium.
iii proven or suspected low compliance to anticoagulation therapy, or failure to reach target INR
iv. unwillingness of patient to provide written informed consent
v. any condition that, in the opinion of the investigator, might interfere with the patient's participation in the study, pose an added risk for the patient, or confound the assessment of the patient

Study procedures

Enrolment
Patients will be enrolled at the clinical institution managing the anticoagulation therapy.
A venous blood sample will be collected for the determination of PNH clone, which will be carried out using flow cytometry according to the procedures described below.
The determination of PNH clone will be centralized and performed in a Center with specific technical skills and experience (Bergamo). Because of the high-sensitivity technique used for the detection of the PNH phenotype, and to improve diagnostic accuracy, we plan to repeat the test with a second blood sample in all patients in whom the first test resulted with FLAER –ve events higher than the 95th percentile.

Information will be collected with regard to: age, sex, race, personal or family history of thromboembolism; family history of PNH; presence of known or documented hereditary or acquired thrombophilic states (antithrombin, protein C, protein S, factor V Leiden, prothrombin G20210A, homocysteine, antiphospholipid antibodies); medical history or new diagnosis of thrombosis of Philadelphia negative myeloproliferative neoplasms; positivity for the JAK2 mutation; presence of other known risk factors for deep venous thrombosis (surgery, trauma, solid cancer, local or systemic infections or inflammatory disorders, hormonal therapy, pregnancy or puerperium).

Endpoints

Primary

- The primary objective of this study is to determine the prevalence of clones with the PNH phenotype in patients with a recurrence/occurrence of thrombosis in patients undergoing primary or secondary antithrombotic therapy.

Secondary

- To determine the cellular lines involved in the expression of the PNH clone and the dimensions of the clone in positive patients;
- To determine the prevalence of PNH clone in thrombotic patients according to the type of antithrombotic therapy
- To determine the prevalence of PNH clone in thrombotic patients according to the site of thrombosis
- To determine the clinical and laboratory correlates of the presence of PNH

Sample size and statistical analysis

Descriptive statistics (mean, standard deviation, median, and range) will be performed for continuous variables (clinical laboratory tests) while count and percentage distribution (n, %) will be calculated for categorical variables. Paired statistical analyses will be used to calculate the variations in size of the PNH clone in patients previously diagnosed positive.

The total prevalence of the PNH clone (for white blood cells ≥ 0.1%), the specific prevalence per cellular group and per patient subgroup as a function of the thrombosis location and the type of antithrombotic therapy will be expressed as a proportion with respect to the total of subjects in the category of reference, with a 95% confidence interval.

Flow cytofluorometry

Peripheral blood is drawn and collected in a *Vacutainer™* with EDTA or heparin.

The expression of *GPI-linked* molecules will be studied within 24 hours with the following monoclonal antibodies (MAb):

- Non-GPI-linked *molecules*: CD45-APC-Cy7, CD33-APC, CD15-PE-Cy7, CD64-PeCy5, CD235a-FITC.
- GPI-*linked molecules*: CD59-PE, CD157-PE.
- GPI-Anchor: FLAER-Alexa-488.

On neutrophil granulocytes counter-coloured with CD45, CD33 and CD15 reactivity for CD157 and FLAER will be evaluated. On monocytes, identified with CD45, CD33 and CD64, the reactivity of CD157 and FLAER will be studied. The CD59 antigen will be assessed on red blood cells in combination with CD-235a. For all the experiments described, a cell "marking-and-lysis" protocol will be used, except for red blood cells, in which lysis will obviously not be performed.

	I	II	III	IV	V	VI
Granulocytes / monocytes	FLAER-Alexa488	CD157-PE	CD64-PeCy5	CD15-PE-Cy7	CD33-APC	CD45-APC-Cy7
Erythrocytes	CD235a-FITC	CD59-PE	Empty	Empty	Empty	CD45-APC-Cy7

Data collection will be performed immediately after completion of the preparation of the sample with a flow cytometer. For each sample, at least $3x10^5$ events will be obtained. The dimension of the clone set as threshold to consider a patient as PNH-positive will be 0.001%.

Methodology

For red blood cells, a 1:500 suspension (e.g., 10 µl of blood in 5 ml of PBS) will be prepared. 50 µl of this suspension will be incubated at 4°C for 20' with appropriate amounts of each monoclonal antibody. After washing in 2 ml of PBS and centrifugation at 2000 rpm for 3 min, the pellet will be suspended in 200-300µl of PBS and acquired at the cytometer.

For white blood cells: 50µl of whole blood (up to 250µl for aplastic patients, depending on the number of neutrophils/µl) will be incubated with monoclonal antibodies. After 20' of incubation at 4°C, the red blood cells will be lysed with 2 ml of ammonium chloride for 15' at room temperature. Subsequently, the sample will be centrifuged at 2000 rpm for 3' and the supernatant, which contains free haemoglobin, will be discarded. The pellet of white blood cells will be suspended in 200µl of PBS acquired at the cytometer.

Cytometric gating strategy

The *gate* on erythrocytes will be performed on physical parameters (FSC and SSC) and completed using CD235a and CD45. The *gate* on leukocytes will be performed using CD45. The gate on granulocytes and monocytes will be performed using CD33 (which has bright expression on monocytes and weak expression on granulocytes) and completed with CD15 and CD64.

Ethical and legal framework
The study protocol will be submitted to Ethic Committees of participating centres. Written informed consent from patients (and, for minors, from parents) will be obtained. The study will be conducted according to Good Clinical Practice (GCP) guidelines within the legal framework of Italian legislation for non-profit studies.

Further developments
Based on the analysis of the results obtained at the end of the study, it may be of interest to proceed with a further investigation, in a larger cohort of patients, whose clinical features will be established accordingly to the conclusions of this preliminary investigation.

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