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Complete List of Authors:	COTLARCIUC, IOANA; Royal Holloway University of London, 1Institute of Cardiovascular Research Marjot, Thomas; Oxford University Hospitals NHS Foundation Trust, Department of Gastroenterology and Hepatology khan, Muhammod; Imperial College London Hiltunen, Sini; Helsinki University Central Hospital, Department of Neurology Haapaniemi, Elena; Helsinki University Central Hospital, Department of Neurology Metso, Tiina; Helsinki University Central Hospital, Department of Neurology Putaala, Jukka; University of Helsinki, Department of Neurological Sciences; Helsinki University Central Hospital, Department of Neurology Zuurbier, Susanna; University of Amsterdam, Department of Neurology Brouwer, Matthijs; University of Amsterdam, Department of Neurology Passamonti, Serena; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Bucciarelli , Paolo; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Pappalardo, Emanuela; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Kosta, Paolo; University of Brescia, Department of Clinical and Experimental Sciences, Neurology Clinic Colombi, Marina; University of Brescia, Department of Molecular and Translational Medicine, Division of Biology and Genetics Canhão, Patricia; University of Lisbon, Department of Neurosciences, Hospital de Santa Maria Tkach , Aleksander; University of Utah, Department of Neurology Santacroce, Rosa; University of Foggia, Department of Clinical and Experimental Medicine Margaglione, Maurizio; University of Foggia, Department of Clinical and Experimental Medicine Favuzzi, Giovanni; .R.C.C.S. Casa Sollievo della Sofferenza S. Giovanni Rotondo, Atherosclerosis and Thrombosis Unit Grandone, Elvira; I.R.C.C.S. Casa Sollievo della Sofferenza, Colaizzo, Donatella; I.R.C.C.S. Casa Sollievo della Sofferenza S. Giovanni Rotondo, Atherosclerosis and Thrombosis Unit Spengos , Konstantinos ; University of Athens School of Medicine Eginition Hospital, Department of Neurology Arauz, Antonio; National Institute of Neurology and Neurosurgery Manuel Velasco Suarez, Stroke Clinic Hodge, Amanda; McMaster University, Pathology and Molecular Medicine.

	Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Ditta, Reina; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Debette, Stéphanie; Universite de Bordeaux, Department of Neurology, Bordeaux University Hospital Pare, Guillaume ; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Ferro, Jose; University of Lisbon, Department of Neurosciences, Hospital de Santa Maria Thijs, Vincent; University of Melbourne, Department of Neurology, Austin Health and Florey Institute of Neuroscience and Mental Health Pezzini , Alessandro ; University of Brescia, Department of Clinical and Experimental Sciences, Neurology Clinic Majersik, Jennifer; University of Utah, Department of Neurology Martinelli , Ida; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Coutinho, Jonathan; University of Amsterdam, Department of Neurology Tatlisumak, Turgut; University of Helsinki, Department of Neurological Sciences; Sahlgrenska University Hospital, Department of Neurology Sharma, Pankaj; Royal Holloway University of London, Institute of Cardiovascular Research
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Towards the genetic basis of cerebral venous thrombosis. The BEAST consortium: a study protocol

Ioana Cotlarciuc[1], Thomas Marjot[2], Muhammad S. Khan[19], Sini Hiltunen[3], Elena Haapaniemi[3], Tiina M. Metso[3], Jukka Putaala[3], Susanna M. Zuurbier[6], Matthijs C. Brouwer[6], Serena M. Passamonti[7], Paolo Bucciarelli[7], Emanuela Pappalardo[7], Paolo Costa[8], Marina Colombi[9], Patrícia Canhão[10], Aleksander Tkach[11], Rosa Santacroce[12], Maurizio Margaglione[12], Giovanni Favuzzi[13], Elvira Grandone[13], Donatella Colaizzo[13], Kostas Spengos[14], Antonio Arauz[15], Amanda Hodge[16], Reina Ditta[16], Stephanie Debette[17], Guillaume Pare[16], José M. Ferro[10], Vincent Thijs[18], Alessandro Pezzini[8], Jennifer J Majersik[11], Ida Martinelli[7], Jonathan M. Coutinho[6], Turgut Tatlisumak[3,4,5], Pankaj Sharma[1], on behalf of the BEAST investigators

[1]Institute of Cardiovascular Research Royal Holloway, University of London (ICR2UL), London, UK

[2]Department of Gastroenterology and Hepatology, University of Oxford, Oxford University Hospitals NHS Trust

[3]Department of Neurology, Helsinki University Central Hospital, Helsinki, Finland

[4]Institute of Neuroscience and Physiology, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

[5]Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden

[6]Department of Neurology, Academic Medical Center, University of Amsterdam, the Netherlands.

[7]A. Bianchi Bonomi Hemophilia and Thrombosis Center, Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, Milan, Italy

[8]Department of Clinical and Experimental Sciences, Neurology Clinic, University of Brescia, Italy

[9]Department of Molecular and Translational Medicine, Division of Biology and Genetics, University of Brescia, Italy

[10]Department of Neurosciences, Hospital de Santa Maria, University of Lisbon, Lisbon, Portugal

[11]Department of Neurology, University of Utah, Salt Lake City, UT, USA.

[12]Medical Genetics, Dept. of Clinical and Experimental Medicine, University of Foggia, Italy.

[13]Atherosclerosis and Thrombosis Unit, I.R.C.C.S. Casa Sollievo della Sofferenza, S. Giovanni Rotondo, Foggia, Italy.

[14] Department of Neurology, University of Athens School of Medicine, Eginition Hospital, Athens, Greece

[15]Stroke Clinic, National Institute of Neurology and Neurosurgery Manuel Velasco Suarez, Mexico City, Mexico.

[16]McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences.

[17]Department of Neurology, Bordeaux University Hospital, Bordeaux University, France

[18]Department of Neurology, Austin Health and Florey Institute of Neuroscience and Mental Health, University of Melbourne, Heidelberg, Victoria, Australia

[19]Department of Restorative Neuroscience, Imperial College London, London, UK

Corresponding author: ioana.cotlarciuc@rhul.ac.uk

Abstract

Introduction

Cerebral venous thrombosis (CVT) is a rare cerebrovascular condition accounting for less than 1% of all stroke cases and mainly affects young adults. Its genetic aetiology is not clearly elucidated.

Methods and analysis

To better understand the genetic basis of CVT, we have established an international biobank of CVT cases, BEAST (Biorepository to Establish the Aetiology of Sinovenous Thrombosis) which aims to recruit highly phenotyped cases initially of European descent and later from other populations. As an initial step, the consortium plans to undertake a genome-wide association analysis of CVT using the Illumina Infinium HumanCoreExome BeadChip to assess the association and impact of common and low frequency genetic variants on CVT risk by using a case-control study design. Furthermore, we aim to identify interactions of genetic variants with several environmental and comorbidity factors which will likely contribute to improve the understanding of the biological mechanisms underlying this complex disease.

Ethics and dissemination

BEAST meets all ethical standards set by local institutional review boards for each of the participating sites. The research outcomes will be published in international peer-reviewed open access journals with high impact and visibility. The results will be presented at both national and international meetings to highlight the contributions into improving the understanding of the mechanisms underlying this uncommon but important disease. This

international DNA repository will become an important resource for investigators in the field of haematological and vascular disorders.

Keywords: cerebral venous thrombosis, ischemic stroke, genetics.

Strengths and limitations of this study

- This study is the largest collaboration on cerebral venous thrombosis conducted to-date and has the advantage that it includes highly phenotyped individuals.
- This is the first study that aims to perform a genome-wide association analysis to assess the association and impact of common and low frequency genetic variants on CVT risk.
- Identifying genetic variants associated with CVT risk will likely contribute to improving our understanding of the biological mechanisms underlying this disease and may lead to the discovery of novel therapeutic targets.
- A potential limitation of the study is the difficulty of recruiting a large number of cases due to the very low incidence and prevalence of this condition. Major efforts are being made to include as many research centres able to investigate this disease across Europe and beyond.

Background

Cerebral venous thrombosis (CVT) is a rare cerebrovascular condition that accounts for <1% of all strokes [1], with an overall annual incidence estimated at 1.32 per 100 000 person-years [2]. CVT commonly affects young adults and is more prevalent in women, accounting for approximately 75% of the adult affected patients [3]. It can lead to mortality or severe morbidity but generally has a good clinical outcome particularly following early identification of less severe cases using advanced imaging [4].

The condition has two broadly different aetiological mechanisms: thrombosis of either cerebral veins with local effects caused by venous obstruction or of the dural sinuses which may cause intracranial hypertension. However, both processes usually occur simultaneously in most patients with thrombosis often present in more than one sinus [1, 5, 6]. Compared to arterial thrombosis, CVT is less frequent in terms of incidence and more variable in its clinical presentation and neuroimaging [7].

The condition has multiple risk factors (Table 1) and presents as a diagnostic and therapeutic challenge given the diversity of symptomatic presentation and variety of putative aetiological factors.

Neither the genetic component of CVT nor its heritability has been widely assessed mainly because of its low incidence and lack of large number of cases. However, there is reasonable evidence to support a genetic predisposition to CVT.

A significant proportion of cases (approximately 13-25%) have no risk factors identified [7, 1] suggesting that undetermined genetic factors may at least partly account for this unexplained risk. Although it is a more rare condition, it does not usually cluster in families and there is no evidence to suggest a Mendelian inheritance.

The genetic component of CVT has so far been assessed mainly by candidate gene studies. Approximately 22% of cases are known to have inherited thrombophilia [1], explaining why most candidate gene studies have assessed mutations associated with this condition such as factor V Leiden and prothrombin G20120A mutation [8]. Other mutations investigated by candidate gene studies have included the MTHFR C677T polymorphism (risk factor for hyperhomocysteinemia) [9], the plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism (risk factor for thrombosis) [10], protein Z G79A polymorphism (involved in formation of blood clots) [11], and Janus Kinase-2 V617F mutation (involved in making hematopoietic cells more sensitive to growth factors) [12]. However, the results from such individual candidate gene studies have been conflicting mainly because of lack of sufficient power due to the low number of cases. One large meta-analysis on 1183 CVT cases and 5189 controls that pooled together results from 26 candidate gene studies highlighted significant associations of factor V Leiden G1691A mutation (OR=2.40; 95%CI=1.75-3.30; $P<10^{-5}$) and prothrombin G20120A mutation (OR=5.48; 95%CI=3.88-7.74; $P<10^{-5}$) in adult populations [13]. Interestingly, this study also found that genes involved in the clotting cascade provide a greater level of thrombosis risk in the cerebral venous circulation compared to its arterial circulation implying a larger genetic liability for CVT compared to

sporadic ischaemic stroke [13]. Moreover, previous studies suggested a stronger genetic component in younger stroke patients compared to older stroke cases providing additional evidence to support a strong genetic susceptibility to CVT [14-16].

Other thrombophilic factors involved in the coagulation pathway that are associated with an increased risk of CVT are: protein C, protein S and antithrombin deficiencies [17]. These prothrombotic factors are also associated with an increased risk of deep vein thrombosis and pulmonary embolism [18, 19] suggesting that all these venous thrombosis conditions may have a common genetic component.

An important characteristic of the disease is the higher prevalence in women. Large epidemiologic studies have confirmed that oral contraceptive (OC) users, particularly users of third-generation OCs, are at increased risk of venous thromboembolism [20-22]. Although contraceptive drugs are an important factor in explaining this gender distribution, genetic factors interacting with pharmacological or environmental determinants may also play a significant role. In addition, very little is known about why the rate of CVT is relatively low given widespread environmental exposures on a population level (e.g. oral contraceptives, sinus infections etc.), suggesting that an underlying background genetic risk may contribute to increasing the incidence of CVT in those with common exposures.

To better understand the genetic basis of CVT, we have established an international biorepository of highly characterized CVT cases, BEAST. The BEAST Consortium includes CVT cases recruited from currently 10 centres across seven countries in Europe, and one each from the USA and Mexico.

Our study aims firstly to assess the association and impact of common and low frequency genetic variants on CVT risk by using a case-control study design and secondly, to identify interactions of genetic variants with several environmental and comorbidity factors which collectively will likely contribute to a better understanding of the biological mechanisms underlying this complex disease.

Methods

Study participants

Cases

The ongoing international BEAST Consortium has to-date recruited DNA and clinical data from 745 CVT patients (aged ≥ 18 years) from 12 research centres located in the following countries: Belgium, Finland, Greece, Italy, the Netherlands, Portugal, UK, USA and Mexico.

In all cases, CVT is confirmed by computed tomography (CT) or magnetic resonance (MR) brain imaging and dedicated venography (CTA (computed tomography angiography), MRA (magnetic resonance angiography), or conventional angiogram). The inclusion criteria for cases are presented in Table 2. Detailed phenotypic data is provided by each participating centre (Table 3).

Due to differences in the genetic structure between the different populations participating to the study [23], cases will be split for genetic association analysis into 4 groups: West European, South European (Italian and Portuguese), Finnish and Mexican cases, to obtain homogenous populations. The US population is all European origin (non-Hispanic white). The results will be presented per ancestral population and then subjected to a pooled meta-analysis of all populations.

Controls

The inclusion criteria for the control population are presented in Table 2.

For the West European CVT cohort, BEAST study will use data from previously genotyped control samples, namely 2,469 British controls from the

1958 British Birth Cohort part of the Wellcome Trust Case Control Consortium (WTCCC) [24, 25].

In addition, we have recruited healthy age- and sex-matched controls numbering 300 Italians for the South European cohort, 230 Finnish for the Finnish cohort, and 100 Mexicans for the Mexican cohort.

Ethical considerations

BEAST meets all ethical standards set by local institutional review boards for each of the participating sites. Written informed consent is obtained for all CVT patients and controls at each participating research centre. Patient confidentiality is protected and patient details are encrypted.

Biological samples

Peripheral blood samples from all participants are collected in EDTA-coated vials or sodium citrate vacutainers using venipuncture. Genomic DNA is extracted from peripheral blood using commercially available DNA isolation kits and stored at -80°C.

Genotyping

Cases

DNA samples for all CVT cases will be processed on the HumanCoreExome BeadChip v1.0 (Illumina, Inc., San Diego, CA) using standard protocols at the Genetic and Molecular Epidemiology Laboratory, McMaster University, Canada.

The Illumina Infinium HumanCoreExome BeadChip contains approximately 240,000 exome focused markers, as well as approximately 240,000 common tagSNP markers. The functional exonic markers include non-synonymous

variants, stop altering variants, splice coding variants and variants located in promoter regions.

Controls

The WTCCC British control sample was genotyped using the HumanExome BeadChip v1.0 (Illumina, Inc., San Diego, CA). The Illumina HumanExome Beadchip includes 247,870 markers focused on protein-altering variants selected from >12,000 exome and genome sequences representing multiple ethnicities and complex traits.

The Finnish controls have already been genotyped using the Illumina Infinium HumanCoreExome BeadChip, while other control samples (Italian and Mexican) will be genotyped with the same array.

Data analysis

We will perform case-control analysis using logistic regression assuming an additive genetic model to assess the association of the genotyped markers with CVT risk. Rigorous quality control procedures will be applied according to the recommended exome chip processing protocol [26].

Population stratification analysis and testing for relatedness will be conducted, and outliers will be removed from analysis. To investigate residual population stratification, genomic inflation factors will be calculated. Quantile-Quantile plots will be performed to assess the quality of the association results. Meta-analysis of the association results for the participating cohorts will be performed using a fixed effect model and inverse variance method of weighted beta coefficients and standard errors from each study. Furthermore, the putative positive findings will be confirmed by replication in independent

cohorts to exclude spurious associations. We are currently collaborating with additional centers to recruit a replication cohort.

We will also assess the interactions of significant polymorphisms with environmental and comorbidity risk factors, severity of clinical presentation and outcome. We will perform sex stratified analysis, adjusting for age, and conduct several comparisons (e.g. between OC users and female non-users, cases with ischemic stroke (IS) and cases without IS, cases with factor V Leiden mutation and cases without the mutation), to highlight the influence of genetic factors between different patient groups.

Sample size and power

Power calculations were performed using the genetic power calculator CaTS [27]. With the current BEAST repository of 745 CVT cases and a total of approximately 3000 controls, the study has 80% power to detect a relative risk (RR) of 1.6 at a significant P-value < 10^{-7} with a population allele frequency of 30%. However, the likely genetic liability of this condition [13] suggests that this power calculation may be conservative.

Discussion

The BEAST consortium is the largest DNA repository of highly characterized CVT cases established to-date. The study aims to improve our understanding of the genetics of CVT by firstly investigating the influence of common and low frequency genetic variants on CVT risk and, secondly, by identifying interactions of genetic variants with environmental and comorbidity risk factors. Comprehensive investigation into the genetics of CVT holds the potential to allow at-risk groups to be identified, as well as disease severity and prognosis to be determined.

In the past several years, the genome-wide association (GWA) approach facilitated by technological developments of high density genome-wide genotyping arrays has been applied for many complex diseases and has been successful in identifying thousands of novel common genetic variants associated with disease risk [28]. However, for ischemic stroke GWA has not been as successful with few genetic variants identified [29-34] likely due to the paucity of power in detecting common genetic variants with small effects which require very large cohorts [35]. Another likely reason for the limited positive results is the clinical heterogeneity of ischemic stroke which is known to be influenced by a heterogeneous collection of disease pathways. Considering that CVT is a rare form of stroke affecting a much younger population and a more clinically homogenous form of stroke, we hypothesize it is likely to be influenced by rare genetic variants with potentially larger effects compared to sporadic stroke.

The use of the Illumina Infinium HumanCoreExome BeadChip, which includes a significant number of exonic markers, will increase the probability of

identifying functional genetic markers with potential large effects. The exome contains a large amount of rare protein-altering variants (missense, nonsense single-base substitutions, insertion–deletions) that are predicted to have functional roles and/or to be deleterious [36, 37] which probably account for a considerable amount of the disease-causing mutations [38]. Thus, although the initial sample size of our CVT cohort is small due to the low prevalence/incidence of this disease, this highly phenotyped clinical and DNA repository of CVT cases has the potential of identifying novel coding functional variants associated with CVT with potential large effects. Increasing the sample size with more CVT cases and replicating any initial findings is clearly necessary and is being directly addressed by the BEAST consortium. Currently, the main limitation of our study is the insufficient power to detect genetic variants with small effects using the genome wide approach due to the sample size of our study but continuous efforts are being made to enhance enrollment. An important advantage of our study is the thorough phenotyping using stringent inclusion and exclusion criteria and collection of large amount of clinical variables enabling not just genetic analysis but also allowing differences of associated risk factors or outcomes to be evaluated.

Establishing a large DNA repository of CVT cases worldwide will help elucidate its genetics leading to an improvement in our understanding of the pathophysiological mechanisms underlying this disease, identifying groups at risk and potentially facilitating the identification of novel therapeutic targets.

List of abbreviations

CVT: cerebral venous thrombosis

BEAST: biorepository to establish the aetiology of sinovenous thrombosis

OR: odds ratio

CI: confidence interval

P: P-value

OC: oral contraceptive

CT: computed tomography

MR: magnetic resonance

CTA: computed tomography angiography

MRA: magnetic resonance angiography

WTCCC: Wellcome Trust Case Control Consortium

IS: ischemic stroke

RR: relative risk

GWA: genome-wide association

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IC was involved in study design, recruitment, contributed to developing the final protocol and drafted the manuscript.

TM was involved in study design, recruitment, contributed to revising the manuscript.

MSK, AH, RD were involved in lab analysis and management of samples.

SH, EH, TMM, JP, SMZ, MCB, SMP, PB, EP, PC, MC, PC, AT, RS, GF, DC were involved in recruitment and lab analysis.

MM, EG, KS, AA, SD, GP, JMF, VT, AP, JJM, IM, JMC, TT are senior investigators who contributed with recruitment and sample collection.

PS conceived the idea and is the principal investigator of BEAST who developed the final protocol and drafted the manuscript.

All authors contributed intellectually to the protocol and draft versions of the manuscript and approved the final manuscript.

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Table 1: Risk factors associated with CVT [3, 7]

Genetic prothrombotic conditions

- Antithrombin deficiency
- Protein C and S deficiency
- Factor V Leiden mutation
- Prothrombin G20120A mutation
- Hyperhomocystinemia caused by MTHFR C677T polymorphism

Acquired prothrombotic states

- Nephrotic syndrome
- Antiphospholipid antibodies
- Pregnancy
- Puerperium

Systemic inflammatory disease

- Systemic lupus erythematosus
- Inflammatory bowel disease
- Wegener's granulomatosis
- Behcet's syndrome
- Sarcoidosis
- Thyroid disease

Systemic infectious disease

- Bacterial: Septicemia, endocarditis, typhoid, tuberculosis
- Viral: Measles, hepatitis, encephalitis, herpes, HIV, cytomegalovirus
- Parasitic: Malaria, trichinosis
- Fungal: Aspergillosis

Head and neck infections

- Extradural: Mastoiditis, sinusitis, otitis, facial cellulitis, osteomyelitis, tonsillitis
- Intradural/parenchymal: Abscess, empyema, meningitis

Hematologic disorders

- Polycythemia (primary and secondary)
- Thrombocythemia
- Anemia (including paroxysmal nocturnal hemoglobinuria)
- Sickle cell disease

Drugs

- Oral contraceptives
- L-asparaginase therapy
- Hormone supplement therapy

Systemic malignancies

- Visceral carcinomas
- Lymphomas
- Leukemia
- Myeloproliferative disease

Central nervous system tumors

- Meningioma, metastases, carcinomatous infiltration

Gastro-intestinal disease

- Ulcerative colitis, Crohn disease

Cardiac disease

- Congenital heart disease, cardiac insufficiency, pacemaker

Mechanical causes and trauma

- Head injury, injury to sinuses or jugular vein, neurosurgical procedures, jugular vein catheterization, lumbar puncture.

Others

- Cerebral infarcts and hemorrhage
- Arteriovenous malformations
- Dural arteriovenous malformation
- Arachnoid cyst
- Internal jugular compression
- Severe exfoliative dermatitis
- Severe dehydration of any cause

Idiopathic

Table 2: Inclusion criteria for CVT cases and controls

Inclusion criteria for CVT cases	Inclusion criteria for controls
Age ≥ 18 years at the time of enrolment	Age ≥ 18 years at the time of enrolment
CVT determined using: - computed tomography (CT) or magnetic resonance (MR) brain imaging - dedicated venography (CTA, MRA, or conventional angiogram)	No previous history of CVT/stroke or any other thrombotic or chronic condition
Patient or relative provision of informed written consent	Provision of informed written consent

Table 3: Phenotypic data provided by each participating centre

Demographic data (age, sex, ethnicity).
Date of CVT diagnosis.
Clinical presentation and symptoms.
Neuroimaging information including sinus/vein involved and extent of oedema, haemorrhage.
Family history of thrombotic or cerebrovascular event.
Thrombophilia screening information: - protein C and S deficiencies, - genetic polymorphisms (factor V G1691A mutation, prothrombin G20210A mutation), - antiphospholipid antibodies, - Lupus anticoagulant, - hyperhomocysteinemia.
Risk factors and associated conditions: - other venous thrombosis, - transient risk factors, - pregnancy, - puerperium, - systemic or brain infections, - systemic inflammatory disease, - hematologic disorders, - drugs (oral contraceptives, L-asparaginase therapy, hormone replacement therapy),

- malignancies, - bowel disease, - cardiac disease, - mechanical causes and trauma (head injury, surgery etc), - severe dehydration of any cause.
Modified Rankin Scale at last follow up.

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Towards the genetic basis of cerebral venous thrombosis. The BEAST consortium: a study protocol

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	Velasco Suarez, Stroke Clinic Hodge, Amanda; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Ditta, Reina; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Debette, Stéphanie; Universite de Bordeaux, Department of Neurology, Bordeaux University Hospital Zedde, Marialuisa; Arcispedale Santa Maria Nuova - IRCCS, Neurology Unit, Stroke Unit Pare, Guillaume ; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Ferro, Jose; University of Lisbon, Department of Neurosciences, Hospital de Santa Maria Thijs, Vincent; University of Melbourne, Department of Neurology, Austin Health and Florey Institute of Neuroscience and Mental Health Pezzini , Alessandro ; University of Brescia, Department of Clinical and Experimental Sciences, Neurology Clinic Majersik, Jennifer; University of Utah, Department of Neurology Martinelli , Ida; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Coutinho, Jonathan; University of Amsterdam, Department of Neurology Tatlisumak, Turgut; University of Helsinki, Department of Neurological Sciences; Sahlgrenska University Hospital, Department of Neurology Sharma, Pankaj; Royal Holloway University of London, Institute of Cardiovascular Research
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Towards the genetic basis of cerebral venous thrombosis.

The BEAST consortium: a study protocol

Ioana Cotlarciuc[1*], Thomas Marjot[2*], Muhammad S. Khan[19], Sini Hiltunen[3], Elena Haapaniemi[3], Tiina M. Metso[3], Jukka Putaala[3], Susanna M. Zuurbier[6], Matthijs C. Brouwer[6], Serena M. Passamonti[7], Paolo Bucciarelli[7], Emanuela Pappalardo[7], Tasmin Patel[1], Paolo Costa[8], Marina Colombi[9], Patrícia Canhão[10], Aleksander Tkach[11], Rosa Santacroce[12], Maurizio Margaglione[12], Giovanni Favuzzi[13], Elvira Grandone[13], Donatella Colaizzo[13], Kostas Spengos[14], Antonio Arauz[15], Amanda Hodge[16], Reina Ditta[16], Stephanie Debette[17], Marialuisa Zedde[20], Guillaume Pare[16], José M. Ferro[10], Vincent Thijs[18], Alessandro Pezzini[8], Jennifer J Majersik[11], Ida Martinelli[7], Jonathan M. Coutinho[6], Turgut Tatlisumak[3,4,5], Pankaj Sharma[1], on behalf of the ISGC (International Stroke Genetics Consortium) and BEAST investigators

* These authors have contributed equally

[1]Institute of Cardiovascular Research Royal Holloway, University of London (ICR2UL), London, UK

[2]Department of Gastroenterology and Hepatology, University of Oxford, Oxford University Hospitals NHS Trust

[3]Department of Neurology, Helsinki University Central Hospital, Helsinki, Finland

[4]Institute of Neuroscience and Physiology, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

[5]Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden

[6]Department of Neurology, Academic Medical Center, University of Amsterdam, the Netherlands.

[7]A. Bianchi Bonomi Hemophilia and Thrombosis Center, Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, Milan, Italy

[8]Department of Clinical and Experimental Sciences, Neurology Clinic, University of Brescia, Italy

[9]Department of Molecular and Translational Medicine, Division of Biology and Genetics, University of Brescia, Italy

[10]Department of Neurosciences, Hospital de Santa Maria, University of Lisbon, Lisbon, Portugal

[11]Department of Neurology, University of Utah, Salt Lake City, UT, USA.

[12]Medical Genetics, Dept. of Clinical and Experimental Medicine, University of Foggia, Italy.

[13]Atherosclerosis and Thrombosis Unit, I.R.C.C.S. Casa Sollievo della Sofferenza, S. Giovanni Rotondo, Foggia, Italy.

[14] Department of Neurology, University of Athens School of Medicine, Eginition Hospital, Athens, Greece

[15]Stroke Clinic, National Institute of Neurology and Neurosurgery Manuel Velasco Suarez, Mexico City, Mexico.

[16]McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences.

[17]Department of Neurology, Bordeaux University Hospital, Bordeaux University, France

[18]Department of Neurology, Austin Health and Florey Institute of Neuroscience and Mental Health, University of Melbourne, Heidelberg, Victoria, Australia

[19]Department of Restorative Neuroscience, Imperial College London, London, UK

[20]Neurology Unit, Stroke Unit, Arcispedale Santa Maria Nuova – IRCCS, Reggio Emilia, Italy

Corresponding author: ioana.cotlarciuc@rhul.ac.uk

Abstract

Introduction

Cerebral venous thrombosis (CVT) is a rare cerebrovascular condition accounting for less than 1% of all stroke cases and mainly affects young adults. Its genetic aetiology is not clearly elucidated.

Methods and analysis

To better understand the genetic basis of CVT, we have established an international biobank of CVT cases, BEAST (Biorepository to Establish the Aetiology of Sinovenous Thrombosis) which aims to recruit highly phenotyped cases initially of European descent and later from other populations. To date we have recruited 745 CVT cases from 12 research centres. As an initial step, the consortium plans to undertake a genome-wide association analysis of CVT using the Illumina Infinium HumanCoreExome BeadChip to assess the association and impact of common and low frequency genetic variants on CVT risk by using a case-control study design. Replication will be performed to confirm putative findings. Furthermore, we aim to identify interactions of genetic variants with several environmental and comorbidity factors which will likely contribute to improve the understanding of the biological mechanisms underlying this complex disease.

Ethics and dissemination

BEAST meets all ethical standards set by local institutional review boards for each of the participating sites. The research outcomes will be published in international peer-reviewed open access journals with high impact and visibility. The results will be presented at both national and international

meetings to highlight the contributions into improving the understanding of the mechanisms underlying this uncommon but important disease. This international DNA repository will become an important resource for investigators in the field of haematological and vascular disorders.

Keywords: cerebral venous thrombosis, ischemic stroke, genetics.

Strengths and limitations of this study

- This study is the largest collaboration on cerebral venous thrombosis conducted to-date and has the advantage that it includes highly phenotyped individuals.
- This is the first study that aims to perform a genome-wide association analysis to assess the association and impact of common and low frequency genetic variants on CVT risk.
- Identifying genetic variants associated with CVT risk will likely contribute to improving our understanding of the biological mechanisms underlying this disease and may lead to the discovery of novel therapeutic targets.
- A potential limitation of the study is the difficulty of recruiting a large number of cases due to the very low incidence and prevalence of this condition. Major efforts are being made to include as many research centres able to investigate this disease across Europe and beyond.

Background

Cerebral venous thrombosis (CVT) is a rare cerebrovascular condition that accounts for <1% of all strokes [1], with an overall annual incidence estimated at 1.32 per 100 000 person-years [2]. CVT commonly affects young adults and is more prevalent in women, accounting for approximately 75% of the adult affected patients [3]. It can lead to mortality or severe morbidity but generally has a good clinical outcome particularly following early identification of less severe cases using advanced imaging [4].

The condition has two broadly different aetiological mechanisms: thrombosis of cerebral veins with local effects caused by venous obstruction and thrombosis of the dural sinuses which may cause intracranial hypertension. However, both processes usually occur simultaneously in most patients with thrombosis often present in more than one sinus [1, 5, 6]. Compared to arterial thrombosis, CVT is less frequent in terms of incidence and more variable in its clinical presentation and neuroimaging [7].

The condition has multiple risk factors (Table 1) and presents as a diagnostic and therapeutic challenge given the diversity of symptomatic presentation and variety of putative aetiological factors.

CVT is a rare manifestation of venous thromboembolism (VTE). Compared to CVT, traditional venous thrombosis manifestations such as deep vein thrombosis (DVT) and pulmonary embolism (PE) are much more common and are diseases of aging [8].

There is a lack of data evaluating the risk of CVT recurrence, as well as whether the risk factors for CVT are similar to those for DVT and PE. One

recent study has found that after a 10 year follow up on patients with DVT and PE only 5.2% developed CVT [9], while for CVT patients only 5.8% developed later on DVT/PE [10]. Therefore, no significant link between CVT and DVT/PE has been found so far.

Interestingly, one study has found no differences in thrombophilia markers between CVT and DVT/PE patients, however the frequency of other risk factors, such as oral contraceptive use, pregnancy or puerperium was significantly different [11]. CVT showed to be more frequent in women, secondary to hormonal factors and less often secondary to trauma, immobilisation or surgery compared to DVT/PE patients [11].

Therefore, it is not clear why CVT occurs less often than DVT/PE, and age dependent differences in the risk profile between CVT and DVT/ PE, as well as genetic factors may play a role in the pathogenesis. Thus, due to its rarity and risk profile, CVT represents a particular form of venous thromboembolism.

Neither the genetic component of CVT nor its heritability has been widely assessed mainly because of its low incidence and lack of large number of cases. However, there is reasonable evidence to support a genetic predisposition to CVT.

A significant proportion of cases (approximately 13-25%) have no risk factors identified [7, 1] suggesting that undetermined genetic factors may at least partly account for this unexplained risk. Although it is a more rare condition, it does not usually cluster in families and there is no evidence to suggest a Mendelian inheritance.

The genetic component of CVT has so far been assessed mainly by candidate gene studies. As CVT is known to be associated with inherited thrombophilia [1], most candidate gene studies have assessed mutations associated with this condition such as factor V Leiden and prothrombin G20120A mutation [12]. Other mutations investigated by candidate gene studies have included the MTHFR C677T polymorphism (risk factor for hyperhomocysteinemia) [13], the plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism (risk factor for thrombosis) [14], protein Z G79A polymorphism (involved in formation of blood clots) [15], and Janus Kinase-2 V617F mutation (involved in making hematopoietic cells more sensitive to growth factors) [16]. However, the results from such individual candidate gene studies have been conflicting mainly because of lack of sufficient power due to the low number of cases. One large meta-analysis on 1183 CVT cases and 5189 controls that pooled together results from 26 candidate gene studies highlighted significant associations of factor V Leiden G1691A mutation (OR=2.40; 95%CI=1.75-3.30; $P<10^{-5}$) and prothrombin G20120A mutation (OR=5.48; 95%CI=3.88-7.74; $P<10^{-5}$) in adult populations [17]. Interestingly, this study also found that genes involved in the clotting cascade provide a greater level of thrombosis risk in the cerebral venous circulation compared to its arterial circulation implying a larger genetic liability for CVT compared to sporadic ischaemic stroke [17]. Moreover, previous studies suggested a stronger genetic component in younger stroke patients compared to older stroke cases providing additional evidence to support a strong genetic susceptibility to CVT [18-20].

Other thrombophilic factors involved in the coagulation pathway that are associated with an increased risk of CVT are: protein C, protein S and antithrombin deficiencies [21]. These prothrombotic factors are also associated with an increased risk of deep vein thrombosis and pulmonary embolism [22, 23] suggesting that all these venous thrombosis conditions may have a common genetic component.

An important characteristic of the disease is the higher prevalence in women. Large epidemiologic studies have confirmed that oral contraceptive (OC) users, particularly users of third-generation OCs, are at increased risk of venous thromboembolism [24-26]. Although contraceptive drugs are an important factor in explaining this gender distribution, genetic factors interacting with pharmacological or environmental determinants may also play a significant role. In addition, very little is known about why the rate of CVT is relatively low given widespread environmental exposures on a population level (e.g. oral contraceptives, sinus infections etc.), suggesting that an underlying background genetic risk may contribute to increasing the incidence of CVT in those with common exposures.

To better understand the genetic basis of CVT, we have established an international biorepository of highly characterized CVT cases, BEAST. The BEAST Consortium includes CVT cases recruited from currently 10 centres across seven countries in Europe, and one each from the USA and Mexico.

Our study aims firstly to assess the association and impact of common and low frequency genetic variants on CVT risk by using a case-control study design and secondly, to identify interactions of genetic variants with several

environmental and comorbidity factors which collectively will likely contribute to a better understanding of the biological mechanisms underlying this complex disease.

Methods

Study participants

Cases

The ongoing international BEAST Consortium has to-date recruited DNA and clinical data from 745 CVT patients (aged ≥ 18 years) from 12 research centres located in the following countries: Belgium, Finland, Greece, Italy, the Netherlands, Portugal, UK, USA and Mexico.

In all cases, CVT is confirmed by computed tomography (CT) or magnetic resonance (MR) brain imaging and dedicated venography (CTA (computed tomography angiography), MRA (magnetic resonance angiography), or conventional angiogram). The inclusion criteria for cases are presented in Table 2. Detailed phenotypic data is provided by each participating centre (Table 3).

Due to differences in the genetic structure between the different populations participating to the study [27], cases will be split for genetic association analysis into 4 groups: West European, South European (Italian and Portuguese), Finnish and Mexican cases, to obtain homogenous populations. The US population is all European origin (non-Hispanic white). The results will be presented per ancestral population and then subjected to a pooled meta-analysis of all populations.

Controls

The inclusion criteria for the control population are presented in Table 2.

For the West European CVT cohort, BEAST study will use data from previously genotyped control samples, namely 2,469 British controls from the

1958 British Birth Cohort part of the Wellcome Trust Case Control Consortium (WTCCC) [28, 29].

In addition, we have recruited healthy age- and sex-matched controls numbering 300 Italians for the South European cohort, 230 Finnish for the Finnish cohort, and 100 Mexicans for the Mexican cohort.

Ethical considerations

BEAST meets all ethical standards set by local institutional review boards for each of the participating sites. Written informed consent is obtained for all CVT patients and controls at each participating research centre. Patient confidentiality is protected and patient details are encrypted.

Biological samples

Peripheral blood samples from all participants are collected in EDTA-coated vials or sodium citrate vacutainers using venipuncture. Genomic DNA is extracted from peripheral blood using commercially available DNA isolation kits and stored at -80°C.

Genotyping

Cases

DNA samples for all CVT cases will be processed on the HumanCoreExome BeadChip v1.0 (Illumina, Inc., San Diego, CA) using standard protocols at the Genetic and Molecular Epidemiology Laboratory, McMaster University, Canada.

The Illumina Infinium HumanCoreExome BeadChip contains approximately 240,000 exome focused markers, as well as approximately 240,000 common tagSNP markers. The functional exonic markers include non-synonymous

variants, stop altering variants, splice coding variants and variants located in promoter regions.

Controls

The WTCCC British control sample was genotyped using the HumanExome BeadChip v1.0 (Illumina, Inc., San Diego, CA). The Illumina HumanExome Beadchip includes 247,870 markers focused on protein-altering variants selected from >12,000 exome and genome sequences representing multiple ethnicities and complex traits.

The Finnish controls have already been genotyped using the Illumina Infinium HumanCoreExome BeadChip, while other control samples (Italian and Mexican) will be genotyped with the same array.

Data analysis

We will perform case-control analysis using logistic regression assuming an additive genetic model to assess the association of the genotyped markers with CVT risk. Rigorous quality control procedures will be applied according to the recommended exome chip processing protocol [30].

Population stratification analysis and testing for relatedness will be conducted, and outliers will be removed from analysis. To investigate residual population stratification, genomic inflation factors will be calculated. Quantile-Quantile plots will be performed to assess the quality of the association results. Meta-analysis of the association results for the participating cohorts will be performed using a fixed effect model and inverse variance method of weighted beta coefficients and standard errors from each study. Furthermore, the putative positive findings will be confirmed by replication in independent

samples to exclude spurious associations. We are currently collaborating with additional centers to recruit a replication sample.

We will conduct a reciprocal look up in genome-wide association studies (GWAS) of other venous thrombosis conditions (DVT/PE) and potentially pooling of analyses from these studies if available.

We will undertake a subgroup analysis of CVT cases with and without history of other venous thrombosis conditions (DVT/PE). We will also undertake a subgroup analysis of CVT cases with and without inherited thrombophilia.

We will assess the interactions of significant polymorphisms with environmental and comorbidity risk factors, severity of clinical presentation and outcome.

The power for gene-environment interactions depends on the magnitude of the environmental exposure frequency. Therefore, the power is higher if the exposure frequency is low and is lower if the exposure is high [31].

We will perform sex stratified analysis, adjusting for age, and conduct several comparisons (e.g. between OC users and female non-users, cases with factor V Leiden mutation and cases without the mutation), to highlight the influence of genetic factors between different patient groups. We will also stratify the data by ischemic stroke (IS) status (cases with IS versus cases without IS).

Sample size and power

Power calculations were performed using the genetic power calculator CaTS [32]. With the current BEAST repository of 745 CVT cases and a total of approximately 3000 controls, the study has 80% power to detect a relative risk (RR) of 1.6 at a significant P-value < 10^{-7} with a population allele frequency of

30%. However, the likely genetic liability of this condition [17] suggests that this power calculation may be conservative.

Discussion

The BEAST consortium is the largest DNA repository of highly characterized CVT cases established to-date. The study aims to improve our understanding of the genetics of CVT by firstly investigating the influence of common and low frequency genetic variants on CVT risk and, secondly, by identifying interactions of genetic variants with environmental and comorbidity risk factors. Comprehensive investigation into the genetics of CVT holds the potential to allow at-risk groups to be identified, as well as disease severity and prognosis to be determined.

In the past several years, the genome-wide association (GWA) approach facilitated by technological developments of high density genome-wide genotyping arrays has been applied for many complex diseases and has been successful in identifying thousands of novel common genetic variants associated with disease risk [33]. However, for ischemic stroke GWA has not been as successful with few genetic variants identified [34-39] likely due to the paucity of power in detecting common genetic variants with small effects which require very large cohorts [40]. Another likely reason for the limited positive results is the clinical heterogeneity of ischemic stroke which is known to be influenced by a heterogeneous collection of disease pathways. Considering that CVT is a rare form of stroke affecting a much younger population and a more clinically homogenous form of stroke, we hypothesize it is likely to be influenced by rare genetic variants with potentially larger effects compared to sporadic stroke.

The use of the Illumina Infinium HumanCoreExome BeadChip, which includes a significant number of exonic markers, will increase the probability of

identifying functional genetic markers with potential large effects. The exome contains a large amount of rare protein-altering variants (missense, nonsense single-base substitutions, insertion–deletions) that are predicted to have functional roles and/or to be deleterious [41, 42] which probably account for a considerable amount of the disease-causing mutations [43]. Thus, although the initial sample size of our CVT cohort is small due to the low prevalence/incidence of this disease, this highly phenotyped clinical and DNA repository of CVT cases has the potential of identifying novel coding functional variants associated with CVT with potential large effects. Increasing the sample size with more CVT cases and replicating any initial findings is clearly necessary and is being directly addressed by the BEAST consortium. Currently, the main limitation of our study is the insufficient power to detect genetic variants with small effects using the genome wide approach due to the sample size of our study but continuous efforts are being made to enhance enrollment. An important advantage of our study is the thorough phenotyping using stringent inclusion and exclusion criteria and collection of large amount of clinical variables enabling not just genetic analysis but also allowing differences of associated risk factors or outcomes to be evaluated.

Establishing a large DNA repository of CVT cases worldwide will help elucidate its genetics leading to an improvement in our understanding of the pathophysiological mechanisms underlying this disease, identifying groups at risk and potentially facilitating the identification of novel therapeutic targets.

List of abbreviations

CVT: cerebral venous thrombosis

BEAST: biorepository to establish the aetiology of sinovenous thrombosis

OR: odds ratio

CI: confidence interval

P: P-value

OC: oral contraceptive

CT: computed tomography

MR: magnetic resonance

CTA: computed tomography angiography

MRA: magnetic resonance angiography

WTCCC: Wellcome Trust Case Control Consortium

IS: ischemic stroke

RR: relative risk

GWA: genome-wide association

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IC was involved in study design, recruitment, contributed to developing the final protocol and drafted the manuscript.

TM was involved in study design, recruitment, contributed to developing the final protocol and revising the manuscript.

MSK, TP, AH, RD were involved in lab analysis and management of samples.

SH, EH, TMM, JP, SMZ, MCB, SMP, PB, EP, PC, MC, PC, AT, RS, GF, DC were involved in recruitment and lab analysis.

MM, EG, MZ, KS, AA, SD, GP, JMF, VT, AP, JJM, IM, JMC, TT are senior investigators who contributed with recruitment and sample collection.

PS conceived the idea and is the principal investigator of BEAST who developed the final protocol and drafted the manuscript.

All authors contributed intellectually to the protocol and draft versions of the manuscript and approved the final manuscript.

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406 **Table 1: Risk factors associated with CVT [3, 7]**

Genetic prothrombotic conditions

- Antithrombin deficiency
- Protein C and S deficiency
- Factor V Leiden mutation
- Prothrombin G20120A mutation
- Hyperhomocystinemia caused by MTHFR C677T polymorphism

Acquired prothrombotic states

- Nephrotic syndrome
- Antiphospholipid antibodies
- Pregnancy
- Puerperium

Systemic inflammatory disease

- Systemic lupus erythematosus
- Inflammatory bowel disease
- Wegener's granulomatosis
- Behcet's syndrome
- Sarcoidosis
- Thyroid disease

Systemic infectious disease

- Bacterial: Septicemia, endocarditis, typhoid, tuberculosis
- Viral: Measles, hepatitis, encephalitis, herpes, HIV, cytomegalovirus
- Parasitic: Malaria, trichinosis
- Fungal: Aspergillosis

Head and neck infections

- Extradural: Mastoiditis, sinusitis, otitis, facial cellulitis, osteomyelitis, tonsillitis
- Intradural/parenchymal: Abscess, empyema, meningitis

Hematologic disorders

- Polycythemia (primary and secondary)
- Thrombocythemia
- Anemia (including paroxysmal nocturnal hemoglobinuria)
- Sickle cell disease

Drugs

- Oral contraceptives
- L-asparaginase therapy
- Hormone supplement therapy

Systemic malignancies

- Visceral carcinomas
- Lymphomas
- Leukemia
- Myeloproliferative disease

Central nervous system tumors

- Meningioma, metastases, carcinomatous infiltration

Gastro-intestinal disease

- Ulcerative colitis, Crohn disease

Cardiac disease

- Congenital heart disease, cardiac insufficiency

Mechanical causes and trauma

- Head injury, injury to sinuses or jugular vein, neurosurgical procedures, jugular vein catheterization, lumbar puncture.

Others

- Cerebral infarcts and hemorrhage
- Arteriovenous malformations
- Dural arteriovenous malformation
- Arachnoid cyst
- Internal jugular compression
- Severe exfoliative dermatitis
- Severe dehydration of any cause

Idiopathic

Table 2: Inclusion criteria for CVT cases and controls

Inclusion criteria for CVT cases	Inclusion criteria for controls
Age ≥ 18 years at the time of enrolment	Age ≥ 18 years at the time of enrolment
CVT determined using: - computed tomography (CT) or magnetic resonance (MR) brain imaging - dedicated venography (CTA, MRA, or conventional angiogram)	No previous history of CVT/stroke or any other thrombotic or chronic condition
Patient or relative provision of informed written consent	Provision of informed written consent

Table 3: Phenotypic data provided by each participating centre

Demographic data (age, sex, ethnicity).
Date of CVT diagnosis.
Clinical presentation and symptoms.
Neuroimaging information including sinus/vein involved and extent of oedema, haemorrhage.
Family history of thrombotic or cerebrovascular event.
Thrombophilia screening information: - protein C and S deficiencies, - genetic polymorphisms (factor V G1691A mutation, prothrombin G20210A mutation), - antiphospholipid antibodies, - Lupus anticoagulant, - hyperhomocysteinemia.
Risk factors and associated conditions: - other venous thrombosis, - transient risk factors, - pregnancy, - puerperium, - systemic or brain infections, - systemic inflammatory disease, - hematologic disorders, - drugs (oral contraceptives, L-asparaginase therapy, hormone replacement therapy),

- malignancies, - bowel disease, - cardiac disease, - mechanical causes and trauma (head injury, surgery etc), - severe dehydration of any cause.
Modified Rankin Scale at last follow up.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	4
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	7-10
Objectives	3	State specific objectives, including any prespecified hypotheses	11
Methods			
Study design	4	Present key elements of study design early in the paper	12
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	12-13
Participants	6	*(*a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	12-13; 23
		(*b*) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	14-15; 24-25
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*13-14*
Bias	9	Describe any efforts to address potential sources of bias	14
Study size	10	Explain how the study size was arrived at	15
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	15
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	14-15
		(*b*) Describe any methods used to examine subgroups and interactions	15
		(*c*) Explain how missing data were addressed	14
		(*d*) If applicable, explain how matching of cases and controls was addressed	12-14
		(*e*) Describe any sensitivity analyses	15
Results			NA (protocol paper)

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	17
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	18
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	17-18
Generalisability	21	Discuss the generalisability (external validity) of the study results	18
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	20

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Towards the genetic basis of cerebral venous thrombosis. The BEAST consortium: a study protocol

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Complete List of Authors:	Cotlarciuc, Ioana; Royal Holloway University of London, 1Institute of Cardiovascular Research Marjot, Thomas; Oxford University Hospitals NHS Foundation Trust, Department of Gastroenterology and Hepatology khan, Muhammod; Imperial College London Hiltunen, Sini; Helsinki University Central Hospital, Department of Neurology Haapaniemi, Elena; Helsinki University Central Hospital, Department of Neurology Metso, Tiina; Helsinki University Central Hospital, Department of Neurology Putaala, Jukka; University of Helsinki, Department of Neurological Sciences; Helsinki University Central Hospital, Department of Neurology Zuurbier, Susanna; University of Amsterdam, Department of Neurology Brouwer, Matthijs; University of Amsterdam, Department of Neurology Passamonti, Serena; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Bucciarelli , Paolo; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Pappalardo, Emanuela; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Patel, Tasmin; Royal Holloway University of London, Institute of Cardiovascular Research Kosta, Paolo; University of Brescia, Department of Clinical and Experimental Sciences, Neurology Clinic Colombi, Marina; University of Brescia, Department of Molecular and Translational Medicine, Division of Biology and Genetics Canhão, Patricia; University of Lisbon, Department of Neurosciences, Hospital de Santa Maria Tkach , Aleksander; University of Utah, Department of Neurology Santacroce, Rosa; University of Foggia, Department of Clinical and Experimental Medicine Margaglione, Maurizio; University of Foggia, Department of Clinical and Experimental Medicine Favuzzi, Giovanni; .R.C.C.S. Casa Sollievo della Sofferenza S. Giovanni Rotondo, Atherosclerosis and Thrombosis Unit Grandone, Elvira; I.R.C.C.S. Casa Sollievo della Sofferenza, Colaizzo, Donatella; I.R.C.C.S. Casa Sollievo della Sofferenza S. Giovanni Rotondo, Atherosclerosis and Thrombosis Unit Spengos , Konstantinos ; University of Athens School of Medicine Eginition Hospital, Department of Neurology Arauz, Antonio; National Institute of Neurology and Neurosurgery Manuel

	Velasco Suarez, Stroke Clinic Hodge, Amanda; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Ditta, Reina; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Debette, Stéphanie; Universite de Bordeaux, Department of Neurology, Bordeaux University Hospital Zedde, Marialuisa; Arcispedale Santa Maria Nuova - IRCCS, Neurology Unit, Stroke Unit Pare, Guillaume ; McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences Ferro, Jose; University of Lisbon, Department of Neurosciences, Hospital de Santa Maria Thijs, Vincent; University of Melbourne, Department of Neurology, Austin Health and Florey Institute of Neuroscience and Mental Health Pezzini , Alessandro ; University of Brescia, Department of Clinical and Experimental Sciences, Neurology Clinic Majersik, Jennifer; University of Utah, Department of Neurology Martinelli , Ida; Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, A. Bianchi Bonomi Hemophilia and Thrombosis Center Coutinho, Jonathan; University of Amsterdam, Department of Neurology Tatlisumak, Turgut; University of Helsinki, Department of Neurological Sciences; Sahlgrenska University Hospital, Department of Neurology Sharma, Pankaj; Royal Holloway University of London, Institute of Cardiovascular Research
Primary Subject Heading:	Neurology
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Towards the genetic basis of cerebral venous thrombosis.

The BEAST consortium: a study protocol

Ioana Cotlarciuc[1*], Thomas Marjot[2*], Muhammad S. Khan[19], Sini Hiltunen[3], Elena Haapaniemi[3], Tiina M. Metso[3], Jukka Putaala[3], Susanna M. Zuurbier[6], Matthijs C. Brouwer[6], Serena M. Passamonti[7], Paolo Bucciarelli[7], Emanuela Pappalardo[7], Tasmin Patel[1], Paolo Costa[8], Marina Colombi[9], Patrícia Canhão[10], Aleksander Tkach[11], Rosa Santacroce[12], Maurizio Margaglione[12], Giovanni Favuzzi[13], Elvira Grandone[13], Donatella Colaizzo[13], Kostas Spengos[14], Antonio Arauz[15], Amanda Hodge[16], Reina Ditta[16], Stephanie Debette[17], Marialuisa Zedde[20], Guillaume Pare[16], José M. Ferro[10], Vincent Thijs[18], Alessandro Pezzini[8], Jennifer J Majersik[11], Ida Martinelli[7], Jonathan M. Coutinho[6], Turgut Tatlisumak[3,4,5], Pankaj Sharma[1], on behalf of the ISGC (International Stroke Genetics Consortium) and BEAST investigators

* These authors have contributed equally

[1]Institute of Cardiovascular Research Royal Holloway, University of London (ICR2UL), London, UK

[2]Department of Gastroenterology and Hepatology, University of Oxford, Oxford University Hospitals NHS Trust

[3]Department of Neurology, Helsinki University Central Hospital, Helsinki, Finland

[4]Institute of Neuroscience and Physiology, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

[5]Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden

[6]Department of Neurology, Academic Medical Center, University of Amsterdam, the Netherlands.

[7]A. Bianchi Bonomi Hemophilia and Thrombosis Center, Fondazione IRCCS Ca'Granda – Ospedale Maggiore Policlinico, Milan, Italy

[8]Department of Clinical and Experimental Sciences, Neurology Clinic, University of Brescia, Italy

[9]Department of Molecular and Translational Medicine, Division of Biology and Genetics, University of Brescia, Italy

[10]Department of Neurosciences, Hospital de Santa Maria, University of Lisbon, Lisbon, Portugal

[11]Department of Neurology, University of Utah, Salt Lake City, UT, USA.

[12]Medical Genetics, Dept. of Clinical and Experimental Medicine, University of Foggia, Italy.

[13]Atherosclerosis and Thrombosis Unit, I.R.C.C.S. Casa Sollievo della Sofferenza, S. Giovanni Rotondo, Foggia, Italy.

[14] Department of Neurology, University of Athens School of Medicine, Eginition Hospital, Athens, Greece

[15]Stroke Clinic, National Institute of Neurology and Neurosurgery Manuel Velasco Suarez, Mexico City, Mexico.

[16]McMaster University, Pathology and Molecular Medicine. Population Health Research Institute and Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences.

[17]Department of Neurology, Bordeaux University Hospital, Bordeaux University, France

[18]Department of Neurology, Austin Health and Florey Institute of Neuroscience and Mental Health, University of Melbourne, Heidelberg, Victoria, Australia

[19]Department of Restorative Neuroscience, Imperial College London, London, UK

[20]Neurology Unit, Stroke Unit, Arcispedale Santa Maria Nuova – IRCCS, Reggio Emilia, Italy

Corresponding author: ioana.cotlarciuc@rhul.ac.uk

Abstract

Introduction

Cerebral venous thrombosis (CVT) is a rare cerebrovascular condition accounting for less than 1% of all stroke cases and mainly affects young adults. Its genetic aetiology is not clearly elucidated.

Methods and analysis

To better understand the genetic basis of CVT, we have established an international biobank of CVT cases, BEAST (Biorepository to Establish the Aetiology of Sinovenous Thrombosis) which aims to recruit highly phenotyped cases initially of European descent and later from other populations. To date we have recruited 745 CVT cases from 12 research centres. As an initial step, the consortium plans to undertake a genome-wide association analysis of CVT using the Illumina Infinium HumanCoreExome BeadChip to assess the association and impact of common and low frequency genetic variants on CVT risk by using a case-control study design. Replication will be performed to confirm putative findings. Furthermore, we aim to identify interactions of genetic variants with several environmental and comorbidity factors which will likely contribute to improve the understanding of the biological mechanisms underlying this complex disease.

Ethics and dissemination

BEAST meets all ethical standards set by local institutional review boards for each of the participating sites. The research outcomes will be published in international peer-reviewed open access journals with high impact and visibility. The results will be presented at both national and international

meetings to highlight the contributions into improving the understanding of the mechanisms underlying this uncommon but important disease. This international DNA repository will become an important resource for investigators in the field of haematological and vascular disorders.

Keywords: cerebral venous thrombosis, ischemic stroke, genetics.

Strengths and limitations of this study

- This study is the largest collaboration on cerebral venous thrombosis conducted to-date and has the advantage that it includes highly phenotyped individuals.
- This is the first study that aims to perform a genome-wide association analysis to assess the association and impact of common and low frequency genetic variants on CVT risk.
- Identifying genetic variants associated with CVT risk will likely contribute to improving our understanding of the biological mechanisms underlying this disease and may lead to the discovery of novel therapeutic targets.
- A potential limitation of the study is the difficulty of recruiting a large number of cases due to the very low incidence and prevalence of this condition. Major efforts are being made to include as many research centres able to investigate this disease across Europe and beyond.

Background

Cerebral venous thrombosis (CVT) is a rare cerebrovascular condition that accounts for <1% of all strokes [1], with an overall annual incidence estimated at 1.32 per 100 000 person-years [2]. CVT commonly affects young adults and is more prevalent in women, accounting for approximately 75% of the adult affected patients [3]. It can lead to mortality or severe morbidity but generally has a good clinical outcome particularly following early identification of less severe cases using advanced imaging [4].

The condition has two broadly different aetiological mechanisms: thrombosis of cerebral veins with local effects caused by venous obstruction and thrombosis of the dural sinuses which may cause intracranial hypertension. However, both processes usually occur simultaneously in most patients with thrombosis often present in more than one sinus [1, 5, 6]. Compared to arterial thrombosis, CVT is less frequent in terms of incidence and more variable in its clinical presentation and neuroimaging [7].

The condition has multiple risk factors (Table 1) and presents as a diagnostic and therapeutic challenge given the diversity of symptomatic presentation and variety of putative aetiological factors.

CVT is a rare manifestation of venous thromboembolism (VTE). Compared to CVT, traditional venous thrombosis manifestations such as deep vein thrombosis (DVT) and pulmonary embolism (PE) are much more common and are diseases of aging [8].

There is a lack of data evaluating the risk of CVT recurrence, as well as whether the risk factors for CVT are similar to those for DVT and PE. One

recent study has found that after a 10 year follow up on patients with DVT and PE only 5.2% developed CVT [9], while for CVT patients only 5.8% developed later on DVT/PE [10]. Therefore, no significant link between CVT and DVT/PE has been found so far.

Interestingly, one study has found no differences in thrombophilia markers between CVT and DVT/PE patients, however the frequency of other risk factors, such as oral contraceptive use, pregnancy or puerperium was significantly different [11]. CVT showed to be more frequent in women, secondary to hormonal factors and less often secondary to trauma, immobilisation or surgery compared to DVT/PE patients [11].

Therefore, it is not clear why CVT occurs less often than DVT/PE, and age dependent differences in the risk profile between CVT and DVT/ PE, as well as genetic factors may play a role in the pathogenesis. Thus, due to its rarity and risk profile, CVT represents a particular form of venous thromboembolism.

Neither the genetic component of CVT nor its heritability has been widely assessed mainly because of its low incidence and lack of large number of cases. However, there is reasonable evidence to support a genetic predisposition to CVT.

A significant proportion of cases (approximately 13-25%) have no risk factors identified [7, 1] suggesting that undetermined genetic factors may at least partly account for this unexplained risk. Although it is a more rare condition, it does not usually cluster in families and there is no evidence to suggest a Mendelian inheritance.

The genetic component of CVT has so far been assessed mainly by candidate gene studies. As CVT is known to be associated with inherited thrombophilia [1], most candidate gene studies have assessed mutations associated with this condition such as factor V Leiden and prothrombin G20120A mutation [12]. Other mutations investigated by candidate gene studies have included the MTHFR C677T polymorphism (risk factor for hyperhomocysteinemia) [13], the plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism (risk factor for thrombosis) [14], protein Z G79A polymorphism (involved in formation of blood clots) [15], and Janus Kinase-2 V617F mutation (involved in making hematopoietic cells more sensitive to growth factors) [16]. However, the results from such individual candidate gene studies have been conflicting mainly because of lack of sufficient power due to the low number of cases. One large meta-analysis on 1183 CVT cases and 5189 controls that pooled together results from 26 candidate gene studies highlighted significant associations of factor V Leiden G1691A mutation (OR=2.40; 95%CI=1.75-3.30; $P<10^{-5}$) and prothrombin G20120A mutation (OR=5.48; 95%CI=3.88-7.74; $P<10^{-5}$) in adult populations [17]. Interestingly, this study also found that genes involved in the clotting cascade provide a greater level of thrombosis risk in the cerebral venous circulation compared to its arterial circulation implying a larger genetic liability for CVT compared to sporadic ischaemic stroke [17]. Moreover, previous studies suggested a stronger genetic component in younger stroke patients compared to older stroke cases providing additional evidence to support a strong genetic susceptibility to CVT [18-20].

Other thrombophilic factors involved in the coagulation pathway that are associated with an increased risk of CVT are: protein C, protein S and antithrombin deficiencies [21]. These prothrombotic factors are also associated with an increased risk of deep vein thrombosis and pulmonary embolism [22, 23] suggesting that all these venous thrombosis conditions may have a common genetic component.

An important characteristic of the disease is the higher prevalence in women. Large epidemiologic studies have confirmed that oral contraceptive (OC) users, particularly users of third-generation OCs, are at increased risk of venous thromboembolism [24-26]. Although contraceptive drugs are an important factor in explaining this gender distribution, genetic factors interacting with pharmacological or environmental determinants may also play a significant role. In addition, very little is known about why the rate of CVT is relatively low given widespread environmental exposures on a population level (e.g. oral contraceptives, sinus infections etc.), suggesting that an underlying background genetic risk may contribute to increasing the incidence of CVT in those with common exposures.

To better understand the genetic basis of CVT, we have established an international biorepository of highly characterized CVT cases, BEAST. The BEAST Consortium includes CVT cases recruited from currently 10 centres across seven countries in Europe, and one each from the USA and Mexico.

Our study aims firstly to assess the association and impact of common and low frequency genetic variants on CVT risk by using a case-control study design and secondly, to identify interactions of genetic variants with several

environmental and comorbidity factors which collectively will likely contribute to a better understanding of the biological mechanisms underlying this complex disease.

Methods

Study participants

Cases

The ongoing international BEAST Consortium has to-date recruited DNA and clinical data from 745 CVT patients (aged ≥ 18 years) from 12 research centres located in the following countries: Belgium, Finland, Greece, Italy, the Netherlands, Portugal, UK, USA and Mexico.

In all cases, CVT is confirmed by computed tomography (CT) or magnetic resonance (MR) brain imaging and dedicated venography (CTA (computed tomography angiography), MRA (magnetic resonance angiography), or conventional angiogram). The inclusion criteria for cases are presented in Table 2. Detailed phenotypic data is provided by each participating centre (Table 3).

Due to differences in the genetic structure between the different populations participating to the study [27], cases will be split for genetic association analysis into 4 groups: West European, South European (Italian and Portuguese), Finnish and Mexican cases, to obtain homogenous populations. The US population is all European origin (non-Hispanic white). The results will be presented per ancestral population and then subjected to a pooled meta-analysis of all populations.

Controls

The inclusion criteria for the control population are presented in Table 2.

For the West European CVT cohort, BEAST study will use data from previously genotyped control samples, namely 2,469 British controls from the

1958 British Birth Cohort part of the Wellcome Trust Case Control Consortium (WTCCC) [28, 29].

In addition, we have recruited healthy age- and sex-matched controls numbering 300 Italians for the South European cohort, 230 Finnish for the Finnish cohort, and 100 Mexicans for the Mexican cohort.

Ethical considerations

BEAST meets all ethical standards set by local institutional review boards for each of the participating sites. Written informed consent is obtained for all CVT patients and controls at each participating research centre. Patient confidentiality is protected and patient details are encrypted.

Biological samples

Peripheral blood samples from all participants are collected in EDTA-coated vials or sodium citrate vacutainers using venipuncture. Genomic DNA is extracted from peripheral blood using commercially available DNA isolation kits and stored at -80°C.

Genotyping

Cases

DNA samples for all CVT cases will be processed on the HumanCoreExome BeadChip v1.0 (Illumina, Inc., San Diego, CA) using standard protocols at the Genetic and Molecular Epidemiology Laboratory, McMaster University, Canada.

The Illumina Infinium HumanCoreExome BeadChip contains approximately 240,000 exome focused markers, as well as approximately 240,000 common tagSNP markers. The functional exonic markers include non-synonymous

variants, stop altering variants, splice coding variants and variants located in promoter regions.

Controls

The WTCCC British control sample was genotyped using the HumanExome BeadChip v1.0 (Illumina, Inc., San Diego, CA). The Illumina HumanExome Beadchip includes 247,870 markers focused on protein-altering variants selected from >12,000 exome and genome sequences representing multiple ethnicities and complex traits.

The Finnish controls have already been genotyped using the Illumina Infinium HumanCoreExome BeadChip, while other control samples (Italian and Mexican) will be genotyped with the same array.

Data analysis

We will perform case-control analysis using logistic regression assuming an additive genetic model to assess the association of the genotyped markers with CVT risk. Rigorous quality control procedures will be applied according to the recommended exome chip processing protocol [30].

Population stratification analysis and testing for relatedness will be conducted, and outliers will be removed from analysis. To investigate residual population stratification, genomic inflation factors will be calculated. Quantile-Quantile plots will be performed to assess the quality of the association results. Meta-analysis of the association results for the participating cohorts will be performed using a fixed effect model and inverse variance method of weighted beta coefficients and standard errors from each study. Furthermore, the putative positive findings will be confirmed by replication in independent

samples to exclude spurious associations. We are currently collaborating with additional centers to recruit a replication sample.

We will conduct a reciprocal look up in genome-wide association studies (GWAS) of other venous thrombosis conditions (DVT/PE) and potentially pooling of analyses from these studies if available.

We will undertake a subgroup analysis of CVT cases with and without history of other venous thrombosis conditions (DVT/PE). We will also undertake a subgroup analysis of CVT cases with and without inherited thrombophilia.

We will assess the interactions of significant polymorphisms with environmental and comorbidity risk factors, severity of clinical presentation and outcome.

The power for gene-environment interactions depends on the magnitude of the environmental exposure frequency. Therefore, the power is higher if the exposure frequency is low and is lower if the exposure is high [31].

We will perform sex stratified analysis, adjusting for age, and conduct several comparisons (e.g. between OC users and female non-users, cases with factor V Leiden mutation and cases without the mutation), to highlight the influence of genetic factors between different patient groups. We will also stratify the data by ischemic stroke (IS) status (cases with IS versus cases without IS).

Sample size and power

Power calculations were performed using the genetic power calculator CaTS [32]. With the current BEAST repository of 745 CVT cases and a total of approximately 3000 controls, the study has 80% power to detect a relative risk (RR) of 1.6 at a significant P-value < 10^{-7} with a population allele frequency of

30%. However, the likely genetic liability of this condition [17] suggests that this power calculation may be conservative.

Discussion

The BEAST consortium is the largest DNA repository of highly characterized CVT cases established to-date. The study aims to improve our understanding of the genetics of CVT by firstly investigating the influence of common and low frequency genetic variants on CVT risk and, secondly, by identifying interactions of genetic variants with environmental and comorbidity risk factors. Comprehensive investigation into the genetics of CVT holds the potential to allow at-risk groups to be identified, as well as disease severity and prognosis to be determined.

In the past several years, the genome-wide association (GWA) approach facilitated by technological developments of high density genome-wide genotyping arrays has been applied for many complex diseases and has been successful in identifying thousands of novel common genetic variants associated with disease risk [33]. However, for ischemic stroke GWA has not been as successful with few genetic variants identified [34-39] likely due to the paucity of power in detecting common genetic variants with small effects which require very large cohorts [40]. Another likely reason for the limited positive results is the clinical heterogeneity of ischemic stroke which is known to be influenced by a heterogeneous collection of disease pathways. Considering that CVT is a rare form of stroke affecting a much younger population and a more clinically homogenous form of stroke, we hypothesize it is likely to be influenced by rare genetic variants with potentially larger effects compared to sporadic stroke.

The use of the Illumina Infinium HumanCoreExome BeadChip, which includes a significant number of exonic markers, will increase the probability of

identifying functional genetic markers with potential large effects. The exome contains a large amount of rare protein-altering variants (missense, nonsense single-base substitutions, insertion–deletions) that are predicted to have functional roles and/or to be deleterious [41, 42] which probably account for a considerable amount of the disease-causing mutations [43]. Thus, although the initial sample size of our CVT cohort is small due to the low prevalence/incidence of this disease, this highly phenotyped clinical and DNA repository of CVT cases has the potential of identifying novel coding functional variants associated with CVT with potential large effects. Increasing the sample size with more CVT cases and replicating any initial findings is clearly necessary and is being directly addressed by the BEAST consortium. Currently, the main limitation of our study is the insufficient power to detect genetic variants with small effects using the genome wide approach due to the sample size of our study but continuous efforts are being made to enhance enrollment. An important advantage of our study is the thorough phenotyping using stringent inclusion and exclusion criteria and collection of large amount of clinical variables enabling not just genetic analysis but also allowing differences of associated risk factors or outcomes to be evaluated.

Establishing a large DNA repository of CVT cases worldwide will help elucidate its genetics leading to an improvement in our understanding of the pathophysiological mechanisms underlying this disease, identifying groups at risk and potentially facilitating the identification of novel therapeutic targets.

List of abbreviations

CVT: cerebral venous thrombosis

BEAST: biorepository to establish the aetiology of sinovenous thrombosis

OR: odds ratio

CI: confidence interval

P: P-value

OC: oral contraceptive

CT: computed tomography

MR: magnetic resonance

CTA: computed tomography angiography

MRA: magnetic resonance angiography

WTCCC: Wellcome Trust Case Control Consortium

IS: ischemic stroke

RR: relative risk

GWA: genome-wide association

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IC was involved in study design, recruitment, contributed to developing the final protocol and drafted the manuscript.

TM was involved in study design, recruitment, contributed to developing the final protocol and revising the manuscript.

MSK, TP, AH, RD were involved in lab analysis and management of samples.

SH, EH, TMM, JP, SMZ, MCB, SMP, PB, EP, PC, MC, PC, AT, RS, GF, DC were involved in recruitment and lab analysis.

MM, EG, MZ, KS, AA, SD, GP, JMF, VT, AP, JJM, IM, JMC, TT are senior investigators who contributed with recruitment and sample collection.

PS conceived the idea and is the principal investigator of BEAST who developed the final protocol and drafted the manuscript.

All authors contributed intellectually to the protocol and draft versions of the manuscript and approved the final manuscript.

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406 **Table 1: Risk factors associated with CVT [3, 7]**

Genetic prothrombotic conditions

- Antithrombin deficiency
- Protein C and S deficiency
- Factor V Leiden mutation
- Prothrombin G20120A mutation
- Hyperhomocystinemia caused by MTHFR C677T polymorphism

Acquired prothrombotic states

- Nephrotic syndrome
- Antiphospholipid antibodies
- Pregnancy
- Puerperium

Systemic inflammatory disease

- Systemic lupus erythematosus
- Inflammatory bowel disease
- Wegener's granulomatosis
- Behcet's syndrome
- Sarcoidosis
- Thyroid disease

Systemic infectious disease

- Bacterial: Septicemia, endocarditis, typhoid, tuberculosis
- Viral: Measles, hepatitis, encephalitis, herpes, HIV, cytomegalovirus
- Parasitic: Malaria, trichinosis
- Fungal: Aspergillosis

Head and neck infections

- Extradural: Mastoiditis, sinusitis, otitis, facial cellulitis, osteomyelitis, tonsillitis
- Intradural/parenchymal: Abscess, empyema, meningitis

Hematologic disorders

- Polycythemia (primary and secondary)
- Thrombocythemia
- Anemia (including paroxysmal nocturnal hemoglobinuria)
- Sickle cell disease

Drugs

- Oral contraceptives
- L-asparaginase therapy
- Hormone supplement therapy

Systemic malignancies

- Visceral carcinomas
- Lymphomas
- Leukemia
- Myeloproliferative disease

Central nervous system tumors

- Meningioma, metastases, carcinomatous infiltration

Gastro-intestinal disease

- Ulcerative colitis, Crohn disease

Cardiac disease

- Congenital heart disease, cardiac insufficiency

Mechanical causes and trauma

- Head injury, injury to sinuses or jugular vein, neurosurgical procedures, jugular vein catheterization, lumbar puncture.

Others

- Cerebral infarcts and hemorrhage
- Arteriovenous malformations
- Dural arteriovenous malformation
- Arachnoid cyst
- Internal jugular compression
- Severe exfoliative dermatitis
- Severe dehydration of any cause

Idiopathic

Table 2: Inclusion criteria for CVT cases and controls

Inclusion criteria for CVT cases	Inclusion criteria for controls
Age ≥ 18 years at the time of enrolment	Age ≥ 18 years at the time of enrolment
CVT determined using: - computed tomography (CT) or magnetic resonance (MR) brain imaging - dedicated venography (CTA, MRA, or conventional angiogram)	No previous history of CVT/stroke or any other thrombotic or chronic condition
Patient or relative provision of informed written consent	Provision of informed written consent

Table 3: Phenotypic data provided by each participating centre

Demographic data (age, sex, ethnicity).
Date of CVT diagnosis.
Clinical presentation and symptoms.
Neuroimaging information including sinus/vein involved and extent of oedema, haemorrhage.
Family history of thrombotic or cerebrovascular event.
Thrombophilia screening information: - protein C and S deficiencies, - genetic polymorphisms (factor V G1691A mutation, prothrombin G20210A mutation), - antiphospholipid antibodies, - Lupus anticoagulant, - hyperhomocysteinemia.
Risk factors and associated conditions: - other venous thrombosis, - transient risk factors, - pregnancy, - puerperium, - systemic or brain infections, - systemic inflammatory disease, - hematologic disorders, - drugs (oral contraceptives, L-asparaginase therapy, hormone replacement therapy),

- malignancies, - bowel disease, - cardiac disease, - mechanical causes and trauma (head injury, surgery etc), - severe dehydration of any cause.
Modified Rankin Scale at last follow up.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	4
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	7-10
Objectives	3	State specific objectives, including any prespecified hypotheses	11
Methods			
Study design	4	Present key elements of study design early in the paper	12
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	12-13
Participants	6	*(*a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	12-13; 23
		(*b*) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	14-15; 24-25
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*13-14*
Bias	9	Describe any efforts to address potential sources of bias	14
Study size	10	Explain how the study size was arrived at	15
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	15
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	14-15
		(*b*) Describe any methods used to examine subgroups and interactions	15
		(*c*) Explain how missing data were addressed	14
		(*d*) If applicable, explain how matching of cases and controls was addressed	12-14
		(*e*) Describe any sensitivity analyses	15
Results			NA (protocol paper)

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	17
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	18
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	17-18
Generalisability	21	Discuss the generalisability (external validity) of the study results	18
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	20

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.