RARE GENETIC VARIANTS ASSOCIATED WITH NEONATAL CEREBRAL ARTERIAL INFARCTION

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1. Project Summary Hypothesis

Vulnerability to sporadic neonatal arterial ischaemic stroke (NAIS) in term newborns is associated with rare de novo variants in genes involved in endothelial rheology, coagulation, and cell adhesion. The WES-trio genetic approach will allow the identification of these variants and the underlying disease-causing pathways in homogeneous subgroups of patients.

Aim

To investigate the contribution of rare genetic variants to neonatal arterial ischaemic stroke risk by targeting the protein-coding regions of the human genome

Secondary aims

a) To identify rare de novo pathogenic variants that might lead to the cause of NAIS. **b**) To discover a major common genetic determinant in subclinical groups which could influence the development of the disease. **c**) To evaluate *in silico* the putative pathogenic impact of the variants identified as possible candidates. **d**) To examine if a specific homogeneous clinical phenotype in NAIS could be determined by a specific genetic determinant. **e**) To decipher the association between the candidate gene and NAIS identifying the relevant biological mechanisms involved.

Design, procedures and methods

Design:

This is a cross-sectional observational national multicentre study for the identification and genetic characterization of NAIS patients. In addition, clinical data are prospectively obtained from each hospital involved in the study.

The study was undertaken with the understood and written consent of the parents of each subject, with the approval of the research ethics committee of each centre, and in compliance with national legislation and the Declaration of Helsinki.

- **Setting**: 6 University Hospitals (Spain) will participate in the study.
- **Patients**:

o **Inclusion criteria**: Newborn infants (> 36 gestational weeks) with symptomatic NAIS with an ischaemic lesion in the territory of the main cerebral arteries confirmed by MRI.

o **Exclusion criteria**: We exclude from the study those infants with NAIS associated with meningitis, vascular malformation, dehydration, congenital metabolic disease, complex heart congenital diseases, arterial dissection, extracorporeal surgery, need for ECMO, or that have genetic thrombophilia: *F5* G1691A, *F2* G20210A, or homozygosity for *MTHFR* C677T genotypes, as well as those whose family refused to participate or continue in the study.

Procedures

- **Family history and maternal diseases**: Family Information is soughed through a specific interview of both parents and from medical charts from the infants and their mothers. The following maternal are being collected: age, weight, and height to calculate body mass index (BMI), hypertension, spontaneous abortions, antiphospholipid syndrome, connective tissue or autoimmune diseases such as lupus or dermatomyositis, thrombocytosis, idiopathic thrombocytopenia, polycystic ovary syndrome, migraine, epilepsy, and ingestion of oral contraceptives. A three-generation pedigree will be obtained for each family regarding and 1) myocardial infarction, 2) pulmonary embolism, 3) cerebrovascular event, and 4) deep vein thrombosis will be sought.
- **Clinical phenotype**: All recruited infants with NAIS will have a careful clinical evaluation during the neonatal period and at discharge. Further, continuous neurophysiological evaluation during the first days after clinical debut, determination of brain damage biomarkers in CSF (neuronal-specific enolase) and studies of multimodal neuroimaging with MRI (T1W, T2W, DWI, ADC, DTI, TOF) during the neonatal period will be performed.
- **MRI Data Acquisition**: MRI will be performed on a General Electric 1.5 T Signal Excite scanner using a specific neonatal head coil following the clinical MRI protocol for neonates with suspected NAIS. The MRI study is performed within 7 days after the occurrence of the neurological symptoms. The protocol as well as the analysis and lesion segmentation has been published.
- **Individual MRI image analysis and brain lesion segmentation**. The lesion segmentation process has been described in detail in a publication of our group. We perform it through the multimodal analysis of MRI images with ITK-Snap software, version 3.0 (http://www.itksnap.org). To simultaneously display images from the different acquisition modalities, the linear transformation flirt function of the FSL suite,

version 5.0 (http://fsl.fmrib.ox.ac.uk), is used. The region competition preprocessing function of ITK-Snap is applied using 5- tissue clustering, 0.5 region competition force, and 0.5 smoothing force. Once satisfactory lesion segmentation is attained in the evolution step, it is manually corrected by simultaneously inspecting its features in all the imaging modalities, as well as its 3D visualization. In this step, a consensus by 3 experts blind to radiological diagnosis will be reached.

- **Outcome**: All patients will be followed, and systematic visits will be performed at ages 6 months, and 1, 2, 3 and 6 years at the different study centres participating in the study. At two years, Bayley Scales of Infant Development (BSID) is performed together with the assessment of motor impairment. The motor impairment is classified with the gross motor function classification scale (GMFCS), according to the child's level of gross motor abilities. In this classification, the subjects are graded in 5 levels: subjects at level 1 walk without limitations and level V subjects need a manual wheelchair. The bimanual fine motor function (BFMF) tool will be also used to assess the ability to grasp, manipulate, and hold objects for each hand separately (level 0, no impairment; level 5, only able to hold or worse with both hands). Each of the subjects enrolled will be evaluated with Wechsler Preschool and Primary Scale of Intelligence™ Fourth Edition (WPPSI IV) at 6 years old. To ensure homogeneity in the clinical assessment of outcome, as well as the absence of bias and database collection, both a neuropsychologist with training also as a speech therapist and a paediatric neurologist, from the coordinator centre, will travel to all study centres involved in the study. They will evaluate the patients blind to their history from the neonatal period, developmental profile and the neuroimaging information (volume and distribution of the ischaemic lesion). The encounter will take place over an entire half-day in a medical setting close to the family residence. Annual newsletters will be sent to keep the families informed of the results of the assessments (neuroimaging and neurodevelopment) and the planned evaluations. Families will be asked to invite their doctor to participate in the study through a letter of intent explaining the characteristics of the study.

This multicentre collaborative study involves an ecological and unique cohort of patients with symptomatic NAIS recruited from several centres following the same protocol. All patients were extensively studied at the clinical, biochemical and neurophysiological levels and particularly with the same MRI protocol.

We obtained, after parents' consent, blood samples from the child who had NAIS and from both parents.

Methodology

- DNA extraction
- Whole-exome sequencing (WES)
- Variant annotation, filtering, and pathogenicity prediction
- Rare *de novo* variants detection
- Identification of NAIS candidate genes
    - o Knowledge-driven analysis
    - o Gene ontology and pathway enrichment
    - o Scoring and prioritization
- Functional studies of the candidate gene identified o Cloning
    - o Western blot
    - o Confocal imaging
- Statistica[l] analyses

2. Results

We have performed whole exome sequencing (WES) in 23 trios (mother-father-child). In total, 1,966 variants from the 23 WES-trio with a mean of 85.5 ± 20.4 per proband met the criteria for further analysis as possible *de novo* variants (dnv). When applying the selection by the list of candidate genes according to the pathophysiology of the NAIS, no proband had variants. This list included genes for cell adhesion, coagulation, inflammation, stroke, and endothelium. Next, we performed the Sanger sequencing of the trio to verify the variants that appeared in the patient but not in their parents. From the 23 probands analyzed, 17 (74%) probands carried at least one dnv that met our filtering criteria. More than one proband carried more than one dnv. In five (21.7%) probands we did not identify variants that met our filtering criteria (synonymous, located in regulatory regions or deep intronic), and one (4.3%) proband did not have DNV identified. Subsequently, to confirm these findings in the five infants without variants meeting the criteria and in the infant without dnv, we conducted a less restrictive reanalysis to ensure that no variant was left out by bioinformatic variables or manual mistakes during the filtering workflow. The re-analysis results corroborated our previous findings. Following the variant analysis, we confirmed 28 unique DNV in 28 genes that were not included in our primary gene list. Furthermore, these 28 identified

genes were unique, meaning that the probands did not share any common gene with dnv variant among them.

Because of these partially negative results, we decided to reanalyze WES-trio data under a new hypothesis of a recessive inheritance associated with NAIS. With the recessive hypothesis, we were able to study compound heterozygous and homozygous variants in the probands. Variant identification and filtering were carried out following the same criteria as in the *de novo* variant identification workflow. From the 23 WES-trio analyzed we found a mean of 10.4 ± 4.8 and 7.3 ± 4.5 per proband of homozygous and compound heterozygous variants, respectively. However, after post-analysis filtering, none of the 168 homozygous and 240 compound heterozygous variants met the filtering criteria or were relevant for further association with NAIS. Subsequently, to identify a major common genetic determinant, we performed gene-interaction network analysis and gene analysis of clinically homogeneous patients according to the affected arterial territory definition of the middle cerebral artery (MCA). None of the approaches had the power to identify genes with a potential relationship with NAIS. However, the use of knowledge-driven analysis for candidate gene identification identified the *PIK3CD* gene and its variant c.1292A>G/p.Gln431Arg, as a possible NAIS candidate gene in a patient with perforant stroke. *PIK3CD* encodes a class I PI3K that binds p85 adapter proteins and GTP-bound RAS. One relevant feature of this protein is that it phosphorylates itself and converts phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5- trisphosphate (PIP3), which recruits and activates downstream proteins containing the pleckstrin homology domains. Additionally, PIK3CD acts as a second messenger via PIP3 that helps to activate the alpha serine/threonine-protein kinase (AKT). PIK3CD forms part of the phosphatidylinositol 3'-kinase (PI3K)-Akt cell-signalling pathway, which is a pathway activated by many types of cellular stimuli or toxic insults and is involved in many molecular mechanisms relevant to NAIS, such as the vascular endothelial growth factor (VEGF), the fluid shear stress, and platelet activation pathways.

To investigate the impact of the mutation on the protein and for further association with NAIS pathophysiology, we have performed *in silico* analyses together with the generation of a PIK3CD wild type and a mutant (c.1292A>G/p.Gln431Arg) mammalian expression vector model. The *in silico* analyses using the DynaMut2, for both the wild type and the p.Gln431Arg variant, demonstrated that, according to the Gibbs Free

Energy (in Kcal/mol), the substitution of the glutamine residue by arginine at the position 431, has a predicted stability change of -LED1I FKC/P RC1IEL1L1K 61K~~CI1~~ 1IZEEFh1I indicates a destabilization of the protein. The mechanism by which this variant destabilizes the protein is due to the alteration of different interactions among residues when compared to the wild type (Gln431). Specifically, these alterations are (1) acquisition of an extra hydrophobic interaction between Arg431 and Tyr484, the substitution of a hydrogen bond with Val483 by two polar interactions, and substitution of a hydrogen bond by an ionic interaction with Asp427 and elimination of two polar interactions.

Regarding the generated expression vectors, both wild type and mutant were transfected in the COS-7 cell line to conduct functional studies based on protein subcellular localization, protein expression, and analysis of the Akt signalling pathway through the pAKT/tAKT ratio quantification. Our results demonstrated that, in the tested conditions, there is no significant difference when comparing the WT with the mutant (p= 0.700 WT vs Gln431Arg, p= 0.609 Mock vs Gln431Arg, p= 0.992 Mock vs WT).

Additionally, the COS-7 cell transfection showed that PIK3CD overexpression presents a punctiform and a gross aggregate pattern as subcellular localization in both $PIK3CD^{WT}$ and $PIK3CD^{Gln431Arg}$. Additionally, quantification of both subcellular localization patterns showed a significant difference when comparing both patterns in both expression vectors. The punctiform aggregate is the more predominant pattern in $PIK3CD^{WT}$ (64%, p= 0.006) whereas the gross aggregate is the main pattern in $PIK3CD^{Gln431Arg}$ (64%, p=0.006). Further analyses and approaches are needed to clarify the pathogenicity impact of this variant in PIK3CD.

The exhaustive genetic analysis of our investigation, under autosomal dominant *de novo* and autosomal recessive inheritance of the coding regions of 23 parent-proband trios with idiopathic NAIS and homogeneous subclinical has not allowed identification of a major common gene or pathway that could explain, or partially explain, the genetic cause of the disease. Moreover, we cannot exclude the possibility that the presence of genetic variations in the genome are the cause of idiopathic NAIS due to the possibility of these variants being located outside the coding regions. To explore this, other

strategies and technological approaches designed to target the non-coding DNA regions are necessary.

In a patient with perforating stroke, the functional study of the candidate gene PIK3CD and its variant c.1292A>G/p.Gln431Arg has shown that this variant could affect the protein function, though further studies and approaches are needed to elucidate whether this gene could be associated with NAIS.

In conclusion, our results do not indicate the need for genetic studies of *de novo* or autosomal recessive inheritance variants in these types of patients. Henceforth it is necessary to consider alternative hypothesis strategies for further studies that might involve an imbalance in susceptibility and protection genetic factors, oligogenic or polygenic inheritance and/or unknown external stimuli with epigenetic influence. Ultimately, further choices of research and additional analyzes are warranted to achieve a better understanding of the genetic aetiology in NAIS and to deepen into other molecular aspects involved in the disease.

3. Relevant and possible future implications

Many investigations based on the application of next-generation sequencing have allowed the identification of several genes and their association with complex diseases. However, the heritability of many diseases is still missing and one cause might be the insufficient evaluation of rare genetic variants. In addition, stroke studies are mainly focused on adults and young adults.

The application of these modern methods of molecular biology to decipher the causes of neonatal major diseases with an impact on neurodevelopment has made it possible to establish the underlying genetic cause in a growing number of disorders initially considered non-genetic. Concomitantly, knowledge of the aetiopathogenesis of major developmental disorders in the neonatal population is relevant because could allow to generation and offer specific and potential therapies that will favour the development of precision medicine as a realistic goal for babies with NAIS. Here is where the pertinence of our study lies. The deciphering of this genetic gap in NAIS together with pursuing a better understanding of the molecular mechanisms involved in idiopathic

NAIS is something that responds to the current yearning for identifying individual profiles, specific therapeutic targets, and safer tailored therapies.
Furthermore, it would allow genomic screening, accurate prognosis and the use of adequate rehabilitation and prophylaxis of comorbidities. Likewise, our study is crucial for families, because it provides reliable prenatal and postnatal counselling.

4. Scientific bibliography generated

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Doctoral thesis: *Application of Next Generation Sequencing to Study the Genetic behind Idiopathic Neonatal Arterial Ischemic Stroke*.
Investigator: Jonathan Olival.
Directors: Alfredo García Alix y Francesc Palau.
Faculty of Biology. Genetics doctorate program. University of Barcelona.
Date of public defence: 9 September 2022.