ANALYSIS OF NEURAL PROGENITOR CELLS IN THE CEREBROSPINAL FLUID OF PRETERM INFANTS WITH PROGRESSIVE POSTHAEMORRHAGIC HYDROCEPHALUS

Developing and optimizing the methods and techniques

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A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Master of Molecular Neuroscience in the Faculty of Health Sciences School of Clinical Sciences
Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Taught Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, this work is my own work. Experimental procedures were carried out firstly with assistance of James Uney's laboratory group, but afterwards by the author. I have identified all material in this dissertation which is not my own work through appropriate referencing and acknowledgement. Where I have quoted or otherwise incorporated material which is the work of others, I have included the source in the references. Any views expressed in the dissertation, other than referenced material, are those of the author.
Summary

Background
Preterm infants are highly vulnerable to intraventricular haemorrhage that originates at the subependymal germinal matrix, the main source of neural precursors until birth. These cells are directly involved in the neuroplasticity of the developing brain. When the ependyma breaks due to increased pressure, blood and neural precursors may drain from the germinal matrix to the cerebrospinal fluid (CSF). Consequent ventricular dilation and increase of intracranial pressure produce progressive posthaemorrhagic hydrocephalus (PPHH). Management includes direct ventricular drainage of the CSF, which can provide a direct source of neural precursor cells (NPC) and the opportunity to study potential endogenous repair mechanisms after brain injury.

Hypothesis and Aims
We hypothesise that NPC are present in the CSF of premature infants with PPHH. Our aim was to develop and optimize the methods and techniques to identify and characterize NPC in the CSF of preterm infants with PPHH.

Methods
Patients included in the study were very premature infants (under 32 weeks of gestational age at birth) with PPHH. Cell component was analysed by different approaches for: immunocytochemistry (ICC) to characterize NPC, digital PCR
(dPCR) to detect NPC gene expression and fluorescence-activated cell sorting (FACS) for detection and isolation of NPC.

Results

Protocols were designed for each technique and for sample handling. ICC showed cells stained with SOX2. Gene expression of SOX2, PAX6, Prom1 and APP was observed by dPCR. Analysis with FACS showed inconclusive results.

Conclusions

Our results showed gene expression of at least four NPC markers and cells stained with one NPC marker. Our and other methods to identify and characterize NPC in CSF need to be further developed and optimized. It should be noted that giving the nature of the study, we were only able to process the first samples from the second month of the start.
Lay Abstract

The production of new neurons in the after birth brain –neurogenesis- relies on stem and progenitor cells which have the capacity of self-renovation and at the same time, transformation into any neuronal cell type. During pregnancy, neurogenesis in the future newborn is highly active in the so-called germinal matrix, a structure flooded by blood vessels located near the brain ventricles. Preterm infants are vulnerable to bleedings in this matrix, which may cause progenitor cells to drain into the fluid filling the ventricles, the cerebrospinal fluid. These bleedings are called intraventricular haemorrhage, and one of the possible negative outcomes is the excess accumulation of fluid in the ventricles, causing head growth and hydrocephalus. To alleviate the pressure inside the head, patients with hydrocephalus after intraventricular haemorrhage undergo direct ventricular drainage of the cerebrospinal fluid, providing researches with first-hand information over the cell component of this fluid. The only study that found neural progenitor cells (NPC) in these patients was published 9 years ago. Our aim was to develop and optimize the methods and techniques to identify and characterize neural progenitor cells in the cerebrospinal fluid of preterm infants with hydrocephalus after intraventricular haemorrhage (PPHH). Patients included in the study were very premature infants (under 32 weeks of gestational age at birth) with PPHH. Cell component was analysed by fluorescent imaging to characterize NPC; polymerase chain reaction (PCR) to detect NPC gene
expression and flow cytometry to detect and isolate NPC. Fluorescent cell imaging showed possible neural progenitor cells with one fluorescent marker of NPC (SOX2 staining). Gene expression of NPC markers was positive for four of them by PCR. Flow cytometry showed inconclusive results. During the study, we designed protocols for sample processing.
Introduction

Neurogenesis and Neural Precursors

The discovery of adult neurogenesis (the production of new neurons in the adult brain), has led to an increasing attention towards Neural Stem Cells (NSC) and its research potential \(^1\). NSC in postnatal human individuals are located in specific neural and glia proliferating enclaves called neurogenic niches \(^2\). These are the subventricular zone (SVZ) near the lateral ventricles, and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus \(^3,4\). The microenvironment of these niches are known to be the main regulator of NSC activity, proliferation and differentiation processes \(^2\).

Neurogenesis relies on self-renewing, multipotent and heterogeneous NSC, which originate Neural Progenitor Cells (NPC) with same potential but less self-renewing capacity. Both cell populations are known as Neural Precursor Cells \(^5\). This precursor cells undergo different steps until becoming fully mature, and are able to divide into different cell types, e.g. neurons, astrocytes and oligodendrocytes. NPC usually migrate from the SVZ to the olfactory bulb (OB) via the rostral migratory system (RMS) or extend their axons from the DG into the CA3 region of the hippocampus. \(^1,6\)
During mammals brain development, neurogenic response after injury can achieve remarkable regeneration in comparison with the adult brain \(^7,8\). In humans, NSC are also present in the SVZ, the ventricular zone (VZ) and the SGZ, usually as specialized astrocytes. Neurogenesis remain highly active in the SGZ but drastically decline in the VZ and the SVZ after infancy. Important to notice, human newborns and infants hold a different new neuron-migratory route directed to the prefrontal cortex. Commonly, pathological conditions activate NSC that are in latent-state (majority of adult stem cell population in the brain). \(^9\)

http://www.nature.com/nature/journal/v414/n6859/full/414112a0.html

Twenty-five years ago, procedures leading to the isolation of neural progenitor cells (NPC) from mammalian brains –including humans- started to materialize \(^{10-12}\). Nowadays, precursor cells are detected and isolated at different stages by different properties including expression of different proteins called markers. These markers, which usually are present in undifferentiated cells, can be intracellular and/or cell-surface markers [Table 1]. Following this reasoning, NPC
and NSC had been isolated not only from neurogenic areas of the CNS but also from non-neurogenic areas like the septum, striatum, cortex and spinal cord. \textsuperscript{1,6,12}

Table 1. Some of the Neural Precursor Cell markers. Examples of markers, which are present in neural stem and progenitor cells. The markers are not specific and are commonly present in other undifferentiated or proliferating cells. On the left column is the name of the marker and on the right a brief description of its importance. All of these markers are currently used to identify Neural Precursor Cells. \textsuperscript{15-26} EMX2: homeobox 2; PAX6: paired-box 6; SOX2: sex determining region Y-box 2; Notch1: Notch homolog 1; Cd133: Prominin 1 or AC133.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>Intermediate filament protein for survival and self-renewal of neural precursor cells</td>
</tr>
<tr>
<td>EMX2</td>
<td>Transcription factor for brain development</td>
</tr>
<tr>
<td>PAX6</td>
<td>Transcription factor for brain development</td>
</tr>
<tr>
<td>SOX2</td>
<td>Transcription factor for precursor cells function</td>
</tr>
<tr>
<td>Notch1</td>
<td>Transmembrane receptor for survival and differentiation of neural precursor cells</td>
</tr>
<tr>
<td>Musashi1</td>
<td>RNA binding protein for posttranscriptional gene regulation</td>
</tr>
<tr>
<td>CD133</td>
<td>Transmembrane protein with not define function</td>
</tr>
</tbody>
</table>
Intraventricular Haemorrhage (IVH): the disruption of normal CSF homeostasis in premature infants.

Supporting the brain and reducing its functional weight by 75%, the CSF circulates from the cerebral ventricles to the subarachnoid space, providing a dynamic medium for adequate perfusion and favourable interaction with the systemic circulation\textsuperscript{27,28}. The CSF –mainly produced in the choroid plexus of the ventricles- is ultra-filtrated plasma, with almost 99% of its composition based on water, and other components like glucose, proteins, electrolytes and cells. Premature infants (less than 37 weeks of gestation) have high values of all of the non-water components of CSF, with percentages more than the normal 1% [Table 1]\textsuperscript{27,29}.

Table 2. Normal CSF composition. The main cellular component is the presence of white blood cells (WBC). Thousands of proteins, some of them unique to this region, compose the CSF\textsuperscript{30-32}. Table from\textsuperscript{27}.

<table>
<thead>
<tr>
<th>Age</th>
<th>WBC/mm\textsuperscript{3} Mean (Range)</th>
<th>Glucose (mg/dL) Mean (Range)</th>
<th>Protein (mg/dL) Mean (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premature infants</td>
<td>9 (0-22)</td>
<td>50 (24-63)</td>
<td>115 (65-150)</td>
</tr>
<tr>
<td>Term newborn</td>
<td>8.2 (0-22)</td>
<td>52 (34-119)</td>
<td>90 (20-170)</td>
</tr>
<tr>
<td>0-4 Weeks</td>
<td>11 (0-35)</td>
<td>46 (36-61)</td>
<td>84 (35-189)</td>
</tr>
<tr>
<td>4-8 Weeks</td>
<td>7.1 (0-25)</td>
<td>46 (29-62)</td>
<td>59 (19-121)</td>
</tr>
<tr>
<td>&gt; 8 Weeks</td>
<td>2.3 (0-5)</td>
<td>61 (45-65)</td>
<td>28 (20-45)</td>
</tr>
</tbody>
</table>

CSF homeostasis may be affected by many factors like infections and genetic disorders. Premature infants, particularly those very premature under 32 weeks\textsuperscript{33}, may present characteristic lesions in the Central Nervous System (CSF),
including intracranial haemorrhage (ICH). There are different types of neonatal ICH, but subarachnoid and intraventricular haemorrhages (IVH) are the ones predominant in preterm infants. The latter is the most severe and almost exclusive to the preterm infant, where it usually accompany respiratory distress 34.

The gross incidence (new cases) of ICH in neonates is 26% 35. In premature infants, ICH is present in at least half of the patients, with the overwhelming majority of cases being IVH (there are some reports indicating incidence of more than 60% of IVH in premature infants) 34,36–38. Yet the figure of 25% in premature infants less than 32 weeks is the most accepted 39. Though the incidence of IVH has declined and the survival of infants with low birth weight has increased in recent years, IVH remains a major cause of long term injury and dead in premature infants. 34

In the preterm infant, the source of the IVH bleeding is restricted to the subependymal germinal matrix (SGM), just next to the lateral ventricles. Crucially, the matrix density from 28 to 32 weeks is highly marked at the head of the caudate nucleus, where the bleeding is most likely to emerge. Nevertheless, the neuropathological consequences of IVH (such as posthaemorrhagic hydrocephalus) tend to be the same in term and preterm infants. 34,36
The SGM is a highly vascularized and proliferative structure that surrounds the ependymal layer of the lateral ventricles and predominates in the head of the caudate nucleus. Up to the sixteenth and twentieth week of gestation, this matrix remains as an important origin of NPC, but then it becomes less eminent and by term, it ceases to exist. Before its involution and throughout the third trimester of pregnancy, this matrix remain as an important provider of glial precursors.\textsuperscript{34,40}

The anterior cerebral, the middle cerebral and the internal carotid arteries irrigate the germinal matrix with terminal branches that build up a capillary bed with irregular endothelial-lined vessels vulnerable to injury, because they just have an endothelial cover. The drainage to the venous system is set by the formation of the terminal vein which receive irrigation from the thalamus, striatum and medullary veins; evacuating at the great cerebral vein of Galen to the systemic circulation.\textsuperscript{34}

The bleeding –most likely to appear in the first days of life and originated in the SGM- enters the lateral ventricles after the rupture of the ependymal layer, pass through the foramina of Magendie and Luschka and stagnant in the basilar cisterns of the posterior fossa [Figure 1]. This stagnation can lead to impairment of the CSF dynamics, principally producing blood clots and obstructing the correct drainage of it.\textsuperscript{34,41}
The pathogenesis of IVH is multifactorial and characterized by three main components: intravascular, vascular and extravascular [Figure 4]. In the premature infant, the autoregulation of the cerebral blood flow is not prepared to deal with important changes that occur in different compromising situations like ventilation problems, asphyxia or labour and delivery. Besides, the microcirculation in the germinal matrix is vulnerable due to deficient vascular lining, high metabolic requirements and the progressive involution itself. Finally, it should take into account the microenvironment surrounding the germinal matrix, where the cell support is deficient and the fibrinolytic activity is increased. Still, the origin of intraventricular haemorrhage is—given the evidence we have at the moment—multifactorial and relies on fibroproliferative reactions, increased tissue repair responses and preceding systemic inflammation. 34,43,44
Table 3. Main factors known to increase the likelihood of IVH in the preterm infant. Depending of individual patients and conditions, some factors may be more important than others are in the pathogenesis of IVH. Text from\textsuperscript{34}. 

<table>
<thead>
<tr>
<th>Intravascular</th>
<th>Vascular</th>
<th>Extravascular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluctuating and elevated cerebral blood flow pattern</td>
<td>Flimsy capillary integrity</td>
<td>Insufficient vascular support</td>
</tr>
<tr>
<td>Platelet and coagulation disturbances</td>
<td>Vulnerability of Matrix capillaries</td>
<td>High fibrinolytic activity in the germinal matrix</td>
</tr>
<tr>
<td>Increase in cerebral venous pressure and pressure-passive circulation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Premature infants in Neonatal Intensive Care Units (NICUs) undergo screening procedures including ultrasonography and lumbar puncture. Nevertheless, the diagnosis relies on the gestational age, the clinical findings and the imaging conclusions all together\textsuperscript{34,45}. The immediate treatment requires the maintenance of an adequate cerebral perfusion and the conservation of hemodynamic blood flow in the brain with ventilation and thermoregulation.

*Progressive Posthaemorrhagic Hydrocephalus (PPHH)*
The neuropathological consequences of IVH include germinal matrix destruction (GMD), periventricular haemorrhagic infarction (PHI) and progressive posthaemorrhagic hydrocephalus (PPHH), in that chronological order [Figure 3]. The aforementioned terminal vein is the main localization of infarctions in these patients, because the vessel confluence forms an angle against the blood flow. The blood accumulated in the SGM forms hematomas, which can obstruct the terminal vein and cause ischemia and infarctions. Although not strictly the same, PPHH is also known as post-haemorrhagic ventricular dilatation (PHVD) in the scientific literature, but PPHH diagnosis depends on intracranial pressure (IP) [46–48].

Table 4. Neuropathological consequences of IVH. Text from Volpe et al. 34. GMD: Germinal Matrix Destruction; PHI: Periventricular Haemorrhagic Infarction; PPHH: Progressive Post Haemorrhage Hydrocephalus.

- **GMD**
  - Consequent destruction of glia precursors and failure in brain development

- **PHI**
  - Asymmetric white matter necrosis predominant in the frontal and parietal lobe.

- **PPHH**
  - Acute hydrocephalus (days after IVH)
  - Subacute-chronic hydrocephalus (weeks after IVH)
PPHH is the final consequence of IVH, due to an obstruction of the arachnoid villi by a blood clot or the shatter of the subarachnoid space in the posterior fossa. The first case produces acute hydrocephalus and the second one subacute-chronic hydrocephalus. Both mechanisms cause obstruction of the normal CSF flow and the consequent fluid accumulation. The fibrinolytic mechanisms of the preterm infant are in general very immature and incapable to cope quickly with clots. In general, up to half of patients with IVH develop PPHH, with four times more likely to develop cognitive and psychomotor impairments than term infants. 

PPHH appears when the ventricles dilate rapidly, with high intracranial pressure and consequent head grow. In the short-term, premature infants with IVH may develop PPHH that can be fatal (10 to 30%) or alternatively they might survive the affection but with important neurological consequences such as cognitive or motor deficits (15 to 75% depending on the amount of bleeding, infarction, newborn age and other factors). By compression and ischaemia, PPHH can injure an already affected brain due to the precedent bleeding. In the presence of PPHH, patients show signs of ventricular dilation, head circumference enlargement, fontanel convection, sagittal suture widening and intracranial pressure clinical signs (seizures, hypoventilation, and bradycardia). 

PPHH treatment is mainly conservative. Close surveillance of head growth, ventricular dilation, clinical presentation and Doppler’s resistance index represent
the therapeutic foundations of PPHH. When close surveillance shows a rapid deterioration and significant increase of the aforementioned parameters (head growth >2 cm per week, ventricular size >1.5 cm), it must be considered the use of ventricular drainage (reservoirs, external and direct drains) and/or ventricular shunt (from ventricles to peritoneum) instead of serial lumbar punctures.  

Spontaneous resolution is high in minor bleedings and low in severe bleedings. Once progressive ventricular dilation is established, spontaneous arrest can occur in 50 to 60% of patients, with the rest in the need of ventricular shunt. These patients may suffer neurodevelopmental deterioration in more than 50% (up to 80%) and cerebral palsy in almost 70% of cases.  

**Antecedents**

Previous work exists in the CSF of preterm infants with PPHH. For instance, high throughput assays show at least 438 differences exist between the proteome of patients with PPHH and healthy newborns. Transform growth factor beta 1 (TGFβ-1), vascular endothelial growth factor (VEGF) and the aminoterminal pro-peptide of type 1 collagen (PC1NP) concentrations are significantly higher in PPHH than healthy neonates and others with different hydrocephalus conditions. Consistently, TGFβ-1 and VEGF levels –along some pro-inflammatory cytokines such as TNF-α and IL-1β- decrease after a few weeks of developing PPHH (something similar has shown with IL-1β and INF-γ).
Those biomarkers could represent an arachnoid fibroproliferative reaction that causes fibrosis related malabsorption and a repair and growth reaction to injury. Other studies show IL-6 as a risk factors for developing IVH. Other pro-inflammatory cytokines like IL-18 and IL-1β are elevated in PPHH, as it happens with IFN-γ. In theory, this findings have support the idea behind anti-inflammatory therapy to protect white matter in PPHH. Other developmental mediators in the brain, like amyloid precursor protein (APP), neural cell adhesion molecule-L1 (L1CAM), brevican (BCAN) and neural cell adhesion molecule-1 (NCAM-1) are also increased in PPHH. Moreover, APP has direct correlation with ventricular size and reflects its changes, showing a potential role as a biomarker of the disease.

In contrast, limited research exists regarding cellular analysis of the CSF of preterm infants with PPHH. Most of the studies show proteome analysis and descriptive efforts for physiopathological understanding and biomarkers potential. One study by Krueger et al. focused on isolating and characterizing NPC in the CSF of these patients. The authors found cells that transcribed NPC markers, which they distinguished by immunocytochemistry, flow cytometry and western blot. This was the first time something similar was done. However, there is no account of the NPC or NSC number in the CSF of these patients, and this one study has not been replicated.
Krueger’s study shows that CSF of preterm infants with PPHH may be a source of NPC. Because of the mechanisms described and the origin of the haemorrhage, precursor cells may drain into the CSF and lose their ability to function normally. That is because the CSF of these patients represents a harmful and detrimental environment for cell proliferation and differentiation. 

17,56,57

**Significance**

PPHH typically occurs between 24 and 37 weeks of gestational age. This period is fundamental for brain development, neurogenesis, precursor differentiation and migration, synaptogenesis, white matter tracts establishment, myelination and cortical structure consolidation 39. As stated, current diagnosis and treatment of IVH and PPHH are limited to conservative measures, but at the same time, consequences are incapacitating and life threatening. Successful and systematic strategies to detect, isolate and characterise neural precursor cells (like NSC and NPC) in the CSF of preterm infants with PPHH is really important. It can help to understand the physiopathology of the affection, it can change the current management approach, it can provide a direct source of stem cells and it could represent the first steps to foresee an autologous stem cell treatment in these very same patients and others with distinct neurological diseases.

**Hypothesis**
Neural progenitor cells (NPC) are present in the cerebrospinal fluid (CSF) of preterm infants with Progressive Posthaemorrhagic Hydrocephalus (PPHH).

**Aims**

Design and optimize the methods to detect, isolate and characterise NPC from the cerebrospinal fluid of preterm infants with progressive posthaemorrhagic hydrocephalus.

Design specific protocols for the different methods to detect, isolate and characterise NPC from the cerebrospinal fluid of preterm infants with progressive posthaemorrhagic hydrocephalus.

**Methods**
Samples were obtained from three very premature infants (born at under 32 weeks of gestational age at birth), with PHVD/PPHH diagnosis, receiving treatment in the Neonatal Intensive Care Units at Southmead and St. Michael's Hospital in the city of Bristol, United Kingdom. The duration of the study was from June to August 2015, and carried out in the laboratory facilities of the Biomedical Sciences building of the University of Bristol. Samples were derived either from implanted reservoirs or from lumbar puncture. Exclusion criteria included genetic abnormalities and ventriculitis. Consent form was obtained from the patient’s parents, reassuring anonymity in the study. All approvals for handling human samples and processed them for this specific study were obtained from the pertinent institutions.

The obtained samples underwent one of the following processes: immediate processing, stabilisation with cellular antigen fixatives, suspension with nucleic acid protection reagents, refrigeration or freezing at -83 °C. Samples processed immediately were analysed by either Immunocytochemistry (ICC), Polymerase Chain Reaction (PCR) or Fluorescence-Activated Cell Sorting (FACS). Samples stabilised for antigen preservation were used for FACS or PCR. Frozen samples and those suspended in RNA protection media were processed for PCR. Samples refrigerated from one to five days were used for PCR or ICC. Prior to analysis, cell count was performed and samples were centrifuged at 1000xg for 6
minutes; the supernatant was stored at -83 °C for further analysis and the cell pellet used for the described procedures.

**Cell Count**

Samples were transferred to 15 ml Falcon Tubes and mixed. A solution of 10 ul of Tryptan Blue 0.4% and 10 ul of the sample was pipetted into a double chamber haemocytometer and observed under the microscope.

**Immunocytochemistry (ICC)**

Two approaches were used for ICC: immediate fixation or cell culture for 7 days.

**Immediate Fixation.** Cell pellet was fixed with 4% paraformaldehyde (PFA), spun down for 2 minutes at 12000xg and resuspended in deionized water. The final solution was treated as a smear and plated in adhesion microscopic slides (Superfrost).

**Cell Culture.** Cell pellet was resuspended in media containing DMEM/F12, 2% B27, 1% penicillin/streptomycin, EGF 20 ng/ml and bFGF 20 ng/ml. Cell suspension was plated in previously coated coverslips (fibronectin/poly-L-ornithine), and fresh EGF and bFGF were added every day. Cells were stained on the day 7. Initial cell spotting was performed with 40 ul of one reservoir sample and incubated for 15 minutes at 37 °C.
Experiments were at room temperature unless otherwise explained. Media was aspirated and wells washed with 400 ul of PBS three times with 10 minutes incubation each time. Cells were fixed into coverslips with 500 ul of 4% PFA in PBS for 15 minutes at 37.5 °C in the incubator and washed with 400 ul of PBS three times. Permeabilisation and blocking were performed with 500 ul of PBS 1x, 10% donkey serum and 0.3% Triton X100 for 45 minutes. Cells were incubated overnight at 2°C with 300 ul of PBS 1x, 1% donkey serum, 0.3% Triton X100 and a dilution of 1:200 of one of the primary antibodies: Anti-Nestin Purified Mouse Monoclonal IgG2A, Anti-Notch-1 Affinity Purified Goat IgG, Anti-Musashi-1 Affinity Purified Goat IgG and Anti-SOX1 Affinity Purified Goat IgG; all from R&D Systems’ Human/Mouse/Rat Neural Progenitor Cell Marker Antibody Panel, Catalog No. SC025. Cells were washed three times with PBS and incubated for one hour in the dark with PBS 1x, 1% donkey serum and a dilution of 1:250 of the secondary antibodies: donkey anti-Goat IgG Alexa Fluor® 568 conjugate and donkey anti-Mouse IgG Alexa Fluor® 568 conjugate. Cells were again permeabilised and blocked for co-staining with 1:2000 of Mouse alpha-tubulin monoclonal antibody and 1:500 of anti-Mouse Alexa Fluor® 488 conjugate with the same protocol as before. Cells were washed with PBS three times. DNA staining was carried out with 200 ul of 1:1000 Hoechst. Cells were washed again and media was mounted with a DABCO/MOWIOL solution for imaging. Cells in Superfrost underwent the procedure described without fixation. For primary antibodies, 1:500 dilution was also tested. One variant was used with the last sample, using 1% BSA in all solutions.
RNA isolation was obtained from samples that were either frozen, refrigerated, fresh (within three hours) or treated with nucleic acid protector reagents (*RNA later*). RNA was isolated using the *RNAqueous Total RNA Isolation Kit* (Ambion). After isolation, RNA yield was measured on the *Spectrophotometer Implen NanoPhotometer™ Pearl*. cDNA was synthesized using the *High Capacity cDNA Reverse Transcription Kit* (Applied Biosystems) and analyzed either by Real Time PCR (qPCR) or digital PCR (dPCR) with *TaqMan® Gene Expression Assays* for NPC markers: Nestin, SOX2 (*sex determining region Y-box 2*), Prom-1 (*prominin-1 gene that express CD133 antigen*), APP (*amyloid-beta precursor protein*), PAX-6 (*paired-box 6*), EMX-2 (*homeobox 2*) and the housekeeping gene GAPDH (*Glyceraldehyde 3-phosphate dehydrogenase*).

**Real Time PCR.** The instrument *QuantStudio™ 3 Real-Time PCR Systems* by *Applied Biosystems* was used for qPCR amplification. The rough data was obtained from the incorporated software.

**Digital PCR.** Chips were loaded and sealed with the *QuantStudio™ 3D Digital PCR Chip Loader* and the *Thermal Cycler ProFlex™ 2x Flat PCR System* was used for amplification. Chip data was analyzed by the *QuantStudio® 3D Digital PCR System*. All instruments from *Applied Biosystems*.
Fluorescence-activated Cell Sorting

Fresh samples and those preserved under a cellular antigen stabilization reagent (TransFix®) were analysed. Cell suspension was centrifuged at 300xg for 10 minutes at room temperature, the supernatant was discarded. Cells were then resuspended in 50 or 100 ul of a previously filtrated buffer containing PBS 1x, 2mM EDTA and 0.5% BSA. Ten microliters of CD133 cell surface marker (CD133/1 (AC133)-PE from Miltenyl Biotec) was added to the suspension and incubated for 10 minutes in the dark at 4 °C. Cells were washed in buffer and centrifuged again, discarding the supernatant. Cells were resuspended in 0.2 or 1 ml of the buffer. All samples were collected in polystyrene test tubes and sealed with laboratory film until analysis. Just before analyzing, 2 ul of DRAQ5 were added to each tube, the cells were flicked gently and incubated for 10 minutes. Then the samples were analysed by FACS. From the second run on, samples were passed through a sterile syringe filter and each sample was divided in two for negative controls.

Data Analysis

IBM SPSS Statistics 21 software, under University of Bristol registration was used for cell count analysis and Microsoft Excel 2013 for analyze data from qPCR. The QuantStudio™ 3D AnalysisSuite™ Software was used online -under
free access supported by LifeTechnologies- for dPCR data analysis. For FACS, Flowing software 2 under free access was used.

Results

From all the 18 samples received at the laboratory, 15 were processed. One was fixed to be further analysed by FACS (P003/1, see Table 4), the other two (P002/2 and P002/3) did not show any cell pellet prior to staining and fixation for FACS. The average cell count for the first patient was 3.5 cells per ul (5.2 mls total) and for the second patient 0.7 cells per ul (3.08 mls total). Count was variable over time, with no specific pattern.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Date</th>
<th>Lab reception</th>
<th>Volume (mL)</th>
<th>Appearance</th>
<th>Origin</th>
<th>Initial step</th>
<th>Cell count/µL</th>
<th>Final method</th>
<th>Results</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P001</td>
<td>1</td>
<td>03-Jul</td>
<td>06-Jul</td>
<td>8.25</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Stabilized by TF</td>
<td>5.4</td>
<td>FACS-CD133</td>
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<td>No internal control</td>
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<tr>
<td></td>
<td>2</td>
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<td>06-Jul</td>
<td>1</td>
<td>Xantochromic</td>
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<td>3</td>
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<td>06-Jul</td>
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<td>Xantochromic</td>
<td>Reservoir</td>
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<td>4</td>
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<td>9.5</td>
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<td>5</td>
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<td>09-Jul</td>
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<td>Reservoir</td>
<td>Mixed in FACS buffer</td>
<td>5</td>
<td>FACS-CD133</td>
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<td></td>
<td>6</td>
<td>07-Jul</td>
<td>09-Jul</td>
<td>3.5</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Mixed in FACS buffer</td>
<td>6</td>
<td>FACS-CD133</td>
<td>Unconclusive</td>
<td>No internal control</td>
</tr>
<tr>
<td>7</td>
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<td></td>
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<tr>
<td></td>
<td>8</td>
<td>10-Jul</td>
<td>13-Jul</td>
<td>3.7</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Pooled</td>
<td>0.9</td>
<td>Fixed immediately ICC protocol</td>
<td>False positives</td>
<td>Not enough cells in the slide</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>11-Jul</td>
<td>13-Jul</td>
<td>4</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Pooled</td>
<td>2.8</td>
<td>Cell culture for ICC 60% cell confluency</td>
<td>Delay culture Antibody dilutions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12-Jul</td>
<td>13-Jul</td>
<td>9.5</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Pooled</td>
<td>6.1</td>
<td>qPCR</td>
<td>RNA &lt; 4 ng/µL</td>
<td>Delay sample qPCR No gene expression process</td>
</tr>
<tr>
<td>P002</td>
<td>1</td>
<td>17-Jul</td>
<td>17-Jul</td>
<td>4</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Kept it cold</td>
<td>1.1</td>
<td>qPCR</td>
<td>RNA &lt; 4 ng/µL. Gene expression of GAPDH</td>
<td>Not enough cells</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18-Jul</td>
<td>20-Jul</td>
<td>4</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Stabilized by TF</td>
<td>.</td>
<td>FACS</td>
<td>Cell pellet lost during process 11 days post stabilization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19-Jul</td>
<td>20-Jul</td>
<td>4.5</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>Stabilized by TF</td>
<td>.</td>
<td>FACS</td>
<td>Cell pellet lost during process 11 days post stabilization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21-Jul</td>
<td>24-Jul</td>
<td>2</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>RNA later 5:1 Pooled</td>
<td>0.6</td>
<td>qPCR</td>
<td>RNA &lt; 4 ng/µL. Gene expression of SOX2, PAX6 and Prom1 Poor RNA preservation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>22-Jul</td>
<td>24-Jul</td>
<td>2</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>RNA later 5:1 Pooled</td>
<td>0.6</td>
<td>qPCR</td>
<td>RNA &lt; 4 ng/µL. Gene expression of SOX2, PAX6 and Prom1 Poor RNA preservation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>23-Jul</td>
<td>24-Jul</td>
<td>2</td>
<td>Xantochromic</td>
<td>Reservoir</td>
<td>RNA later 5:1 Pooled</td>
<td>0.6</td>
<td>qPCR</td>
<td>RNA &lt; 4 ng/µL. Gene expression of SOX2, PAX6 and Prom1 Poor RNA preservation</td>
<td></td>
</tr>
<tr>
<td>P003</td>
<td>1</td>
<td>18-Jul</td>
<td>20-Jul</td>
<td>3</td>
<td>Brown</td>
<td>Lumbar P.</td>
<td>Stabilized by TF</td>
<td>.</td>
<td>Stained with CD133 CD25 and CD234</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20-Jul</td>
<td>20-Jul</td>
<td>6.5</td>
<td>Brown</td>
<td>Lumbar P.</td>
<td>Kept it cold</td>
<td>10,000</td>
<td>RNA isolation 12.4 ng/µl, A260/A280=1.937 Stored at -83</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24-Jul</td>
<td>24-Jul</td>
<td>5</td>
<td>Brown</td>
<td>Lumbar P.</td>
<td>Cell Culture for ICC Up to 80% of cell confluency</td>
<td>110</td>
<td>Cell Culture for ICC Culture too long Dilution optimization</td>
<td>Confluency lost at day 7</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- **Xantochromic** and **Brown** refer to the color of the sample.
- **Lumbar P.** indicates the location of the sample.
- **Stabilized by TF** indicates the method used to stabilize the sample.
- **Cell Culture for ICC** indicates the method used for cell culture for ICC.
- **Fixed with PFA 4%** for FACS indicates the fixation method used for FACS.
- **Panel not ready** indicates that the panel was not ready for analysis.
- **No internal control** indicates that there was no internal control.
- **No positive control** indicates that there was no positive control.
- **False positives** indicates false positives were observed.
- **Delay sample** indicates that the sample was delayed.
- **Dilution optimization** indicates that dilution optimization was performed.
Table 5. Summary of procedures in the samples from three patients. On the left are the sample number, the date of sample acquisition in the hospital (Date) and laboratory reception, volume and in ml and appearance. In the middle are the origin of the sample, the initial procedure and the manual cell count. On the right are the methods, results and personal observations of the results. Observations are examined in the Discussion section. CD133 refers to the cell surface marker used to identify neural precursor cells. Xantochromic: yellowish coloration as explain in the text. TF: TransFix; FACS: Fluorescence-activated cell sorting; qPCR: Real Time PCR; dPCR: Digital PCR; ICC: Immunocytochemistry.

As shown in Table 4, samples from two patients were obtained from reservoirs, implanted instruments to continuously drain CSF. Lumbar puncture was performed in patient 3. The use of a cellular stabilization agent like TransFix was done to preserve the antigen integrity for FACS, as we did for RNA with the reagent RNAlater for PCR. We used fresh samples for cultures.

Sample handling

The following protocols were designed to handle each sample correctly and efficiently:

Table 5. General Protocol for Sample Handling. If the sample can be processed in less than an hour, Protocol 1 should be applied. If not, Protocol 2 should be used. Protocol 2 is applicable within 5 days, with progressive poorer outcomes over time.
Table 6. Protocol 1. If the sample can be processed in less than hour from acquisition, it must be transported in ice. Cell count, centrifugation and supernatant storage should be performed first. The sample can be processed immediately, culture or preserved with RNAlater. The latter could be done in the Hospital. After RNA isolation, sample can be frozen or cDNA can be synthesized. qPCR should be used when RNA concentration is unknown, serial dilutions with a housekeeping gene will optimize the analysis.

<table>
<thead>
<tr>
<th>First Step</th>
<th>Second Step</th>
<th>Third Step</th>
<th>Fourth Step</th>
</tr>
</thead>
</table>
| Transport at 2 °C  
Cell count  
Centrifugation | RNA stabilization  
and storage at 4 °C  
up to 1 month | RNA isolation | Is the RNA concentration known? |
| Supernatant storage at -80 to -90 °C | 5 days  
cell culture | No | Yes |
| | Immediate  
processing | qPCR  
with different dilutions | dPCR |
Table 7. **Protocol 2.** Samples processed after 1 hour should be kept cold. After cell count, samples could undergo culture, antigen stabilization or staining for FACS. Cellular antigen stabilization can be obtained with TransFix. Only when cell culture is performed, centrifugation and supernatant storage should be done. If TransFix applied, samples can be kept cold for up to 8 days. If FACS analysis is not possible within that time, staining and fixation should be done to preserve cellular integrity.

<table>
<thead>
<tr>
<th>First Step</th>
<th>Second Step</th>
<th>Third Step</th>
<th>Fourth Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport at 2 °C</td>
<td>Cell culture</td>
<td>Centrifugation</td>
<td>ICC</td>
</tr>
<tr>
<td>Cell count</td>
<td>for 5 days</td>
<td>Supernatant storage at -80 to -90 °C</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular antigen stabilization</td>
<td>Storage at 2 to 8 °C up to 8 days</td>
<td>Is FACS feasible within 8 days?</td>
<td></td>
</tr>
<tr>
<td>Process right</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>away with FACS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>stain the cells and fix them for up to 1 month</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Process the sample 3 hours prior to analysis</td>
<td></td>
</tr>
</tbody>
</table>

**Immunocytochemistry (ICC)**

For the ICC, we wanted to compare between immediate fixation and cell culture as described before. The result showed that immediate fixation produced a big cell dispersion in adherent slides. Not enough cells were recovered in the process, and just one slide showed meaningful results for *SOX2* staining.
Figure 2. Cells processed by immediate fixation and co-stained with Anti-SOX2 and Anti-Alpha-tubulin under 20-x magnification. A: Hoechst DNA dye shows the presence of cell-like particles. B: Alpha-tubulin staining cellular particles. C: SOX2 staining. D: Overlay showing on the right and at the bottom, one apparent positive cell for SOX2.

For the cultured cells, they were grown in vitro for 7 DIV (days in vitro) before being fixed in 1% PFA. The results showed that cell culture obtained from two samples had better cell confluence than the ones with immediate fixation. Cell culture from a reservoir sample showed 60% of cell confluence on day 5, whereas lumbar puncture (LP) sample showed 80% on the same day. Cell confluence progressively decay until analysis on day 7.
Figure 4. Fixed cells on 7 DIV from reservoir sample. Control coverslip showing Hoechst staining.

Figure 5. Fixed cells on 7 DIV from reservoir sample P001/8-9-10. From left to right and up to bottom, Hoechst dye, Alpha-Tubulin, SOX2 and overlay. 20-x magnitude.

Figure 6. Fixed cells on day culture No.7 from reservoir sample P001/8-9-10. A: from left to right and up to bottom, Hoechst dye, Alpha-Tubulin, SOX2 and overlay. 40-x magnitude. B: Overlay 40-x magnitude for SOX2.
Figure 7. Fixed cells on 7 DIV from reservoir sample P001/8-9-10. From left to right and up to bottom, Hoechst dye, Alpha-Tubulin, SOX2 and overlay. 20-x magnitude.

**Polymerase Chain Reaction (PCR)**

RNA yield was successfully measured in one sample, with 12.4 ng/ul. The other samples had less than 4 ng/ul but the yield was undetected. cDNA levels were not correspondent with the levels mentioned, as qPCR analysis with cDNA levels as a reference was highly inaccurate. Cellular stabilization agents were not effective for RNA preservation.

Real Time PCR showed adequate expression for the housekeeping gene GAPDH (CT values around 17) after serial trials adjusting the cDNA concentration at 49.6 ng/ul per well. No further qPCR analysis could be done. Digital PCR in one sample obtained from reservoir and preserved in RNAlater, showed significant gene expression compared to GAPDH for APP, PAX6, SOX2 and Prom1.
Figure 9. Absolute quantification of gene expression from two reservoir samples P001/8-9-10 (1) and P002/1 (2). PAX6, SOX2 and Prom1 probes showed more than 2000 copies per ul in sample P002/1 (2). GAPDH in sample P001/8-9-10 (1) showed 188 copies per ul. Target precision was 90%. Obtained precision was below 30% with a confidence level of 95%.
Figure 10. Absolute quantification of gene expression from two reservoir samples P001/8-9-10 (1) and P002/I (2). APP probe showed more than 200 copies per ul in sample P002/I (2). GAPDH in sample P001/8-9-10 (1) showed 188 copies per ul. Nestin and EMX1 showed less than 100 copies per ul. Target precision was 90%. Obtained precision was below 30% with a confidence level of 95%.

Fluorescence-Activated Cell Sorting (FACS)

CD133+ cells were not found. Around 400,000 events per sample were recorded. Background levels were adequate.

Discussion

The aim of this project was to detect, isolate and characterise NPC from the CSF of preterm infants with PPHH. Currently there is only one published study on this
topic; by Krueger et al\textsuperscript{17}, where the author described successful NPC identification mainly by PCR, cell culture and flow cytometry. Even though we did not follow all of the methods published in that study, we decided to incorporate some of his methods as part of our experimental design. We modified them according to our laboratory environment and our own aim, but we could not replicate his methods exactly in the time we had. Nevertheless, we used all of his target genes for PCR and an almost exact method for cell culture.

The CSF is clear and crystal under basal conditions. In the presence of haemorrhage, the CSF becomes xantochromic (yellow discoloration due to the breakdown of red blood cells and the release by-products such as bilirubin). The CSF in haemorrhage shows evident and bounteous amounts of red blood cells, elevated protein content, xantochromia and hypoglycorrhachia (low glucose levels). \textsuperscript{34}

Immediately upon receiving the samples, we performed cell counting by using a haemocytometer. We obtained cell counts lower than those values by Krueger et al, (n.b. his counting method was not specified) \textsuperscript{17}. Even though it was a clinical study, we were able to analyse more than 80\% of the samples from three patients treated in Southmead and St. Michael’s Hospital in Bristol. We could not process two samples because we had lost the cell component when trying to prepare them for FACS. The other one is stained with CD133, CD25 and CD234
and fixed, which represent the first future step for the NPC cell surface marker panel design.

An average of 10,178 cells per sample were collected from patients with reservoirs, but a high cell number was collected from one patient that underwent lumbar puncture (LP). Even though we expected the cell number to decrease over time, from the 9 samples of the first patient we did not observe any pattern. We would attribute these results to the delay in obtaining and analysing some samples, but that does not explain why some samples analysed after more than one day had more cells than previous samples analysed on the same day of acquisition. Another reason could relate to the red blood cell (RBC) component, but most of the samples were xanthochromic (indicating RBC degradation).

In contrast, the fluorescence-activated Cell Sorting (FACS) results showed a lot more events compared to our cell count. However, these events does not represent an absolute cell quantification. We used CD133 cell surface marker with our first experiment with FACS to identify NPC, but we did not have a positive control to analyse the correspondent threshold accurately. To overcome this issue, a detail and multi-step cell surface marker panel design must be done. At the moment we are trying to use CD24 and CD234 to discriminate epithelial cells, ependymal cells and the white blood cell component.
Add about FACS: problems: slide 55 i dont know a lot of things of the cell population...i dont have positive controls...stain index...overlapping losing cells while washing...viability problems: slide 55 i dont know a lot of things of the cell population i dont have positive controls stain index overlapping losing cells while washing viability

explain why use a panel of markers and not just one marker

In order to characterize NPC from the mixed cell population, ICC was performed. Immediate fixation (with no cell culturing) failed to detect NPCs. This could be due to low cell number or that this method requires further optimization. The alternative method was by growing the cells for 7 DIV, as was carried out by Krueger et al (2006 where cell confluence was achieved. Our observation was that the cells started to detach from coverslips after DIV 5. As we did not expected this loss, we did not have the time to do ICC on DIV 5. Nevertheless, we obtained at least three images suggesting staining of NPC. All the antibodies used for ICC are known for being NPC markers (Nestin, Notch-1, Musashi-1, SOX2)\textsuperscript{12}. We cannot confidently conclude we have NPC from CSF of these...
patients because we did not have a positive control from similar samples. Another problem was that primary antibody dilution is not yet optimised and our results could easily be false positives.

Another option for detecting NPC is by PCR (qPCR and dPCR). In that regard, the best approach for RNA isolation is the immediate one. The only RNA concentration that could be measured confidently was derived from LP, but this raised a lot of questions, especially related with contamination. Nevertheless, because the patient did not have a complete obstruction, we are confident the sample represented the whole CSF composition. To overcome the concentration problem, cDNA was used with different dilutions for qPCR, using a housekeeping gene (GAPDH).

\[ A_{260}/A_{280} = 1.937 \]

With dPCR, we were able to detect gene expression of at least four markers: SOX-2, PAX-6, Prom1 and APP. The first three are –as stated- well known markers of NPC (Prom1 gene express CD133 protein), but APP expression is intriguing. We included this probe, because previous reports showed that APP protein is directly associated with ventricular size in preterm infants with PPHH^{55}, showing its potential as a disease biomarker. Given our results, we think dPCR was the right tool to use for our samples, because it analyses absolute copy number without the need for standard curves allowing the analysis of low amounts of RNA. For us, the results from gene expression, where 4 NPC
markers could be detected in significant amount, were the most promising of our study.

It should be noted that expression of the housekeeping gene GAPDH was obtained from a different sample that the one that showed the significant gene expression from the 4 NPC markers. Information from the chip with GAPDH was lost during the loading process. However, this do not change the significance of the results because dPCR measures absolute quantification.

Musashi kaneko 2000
Notch 1 zhou 2010 and oishi 2004
Cd133 peh 2009 mizrak 2008
Nestin neovascularization Suzuki 2010 park 2010
EMX1 that gives interneurons and PAX6 wei 2011
Emx1 yoshida
Sox2 universal neural stem cell marker (self renewal and multipotential)…..ellis 2004 ………..Meng li 1998
Pax-6 zhang 2010 y kallur 2008

Reviewing the cerebrospinal fluid (CSF) composition and the release of different signal molecules from cells of the SVZ, the ependyma and the choroid plexus, this fluid might function as a neurogenic niche and as a chemo repulsive gradient
origin for SVZ neuroblast migration\textsuperscript{61–64}. The neurogenic potential of CSF decreases progressively throughout development and suffer dynamic changes in an age dependent manner from embryonic stages to adulthood, mostly because of its own physicochemical properties like the protein composition. The CSF - above all in premature infants with PPHH- is in direct contact with the germinal matrix and with the apical protein complexes of progenitor cells in the neonate, showing its neurogenic capacity. \textsuperscript{61,63,65–68}

at tee end say that even i mentioned npc may lose their function, they might as well still functioning and be affected by continuous withdrawal

It is still under debate, if after injury (like haemorrhage), ependymal cells sheathing the ventricles can be recruited and act as a physiological reservoir to undertake the role of stem cells\textsuperscript{21,69,70}. This could potentially explain our findings in gene expression without the correspondent cell staining. Nevertheless, we know the loss of ependyma can lead to impaired neurogenesis and hydrocephalus, consequently expressing poor SVZ cell proliferation and impaired neuroblast migration redirected to the ventricles. \textsuperscript{49}. It is therefore understandable, that this work will continue in the future.

Problems we incurred were not knowing the amount or stage of progenitor cell development in the CSF of premature infants with PPHH. Among other limitations was the inexperience in handling the samples and the fact they only became available in the last month of the project. Statistics parameters could not
be analysed because this was a pilot experiment looking for the best ways to deal with our samples in order to achieve our long-term objective.

**Conclusions**

We have managed to design specific protocols to identify and characterise Neural Progenitor Cells in premature infants with PPHH. We found gene expression of four NPC markers, laying the foundation for further research optimisation and validation.

**Perspectives**

Digital PCR is ideal to identify NPC in CSF, due to the low amount of cells and RNA yield. FACS analysis could potentially represent the best way to isolate NPC. Characterisation of NPC could be achieved after isolation and specific culture systems such as Neurosphere assays.

Future studies should optimise all the analysed methods and validate them with different comparisons. In general, sample availability will remain because CSF from preterm infants with PPHH is continuously drained and discarded. Once the right methods and techniques are designed, NPC from these patients can represent a huge resource for stem cell research in therapeutics, for neurodegenerative disorders and neurological sequelae of the very same patients with PPHH.
References


49. Rodríguez EM, Guerra MM, Vío K, et al. A cell junction pathology of neural stem cells leads to abnormal neurogenesis and hydrocephalus *.


