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The NESHIE and CP Genetics Resource (NCGR): A database of genes and variants reported in neonatal encephalopathy with suspected hypoxic ischemic encephalopathy (NESHIE) and consequential cerebral palsy (CP)

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ABSTRACT

Neonatal encephalopathy (NE) with suspected hypoxic ischaemic encephalopathy (HIE) (NESHIE) is a complex syndrome occurring in newborns, characterised by altered neurological function. It has been suggested that genetic variants may influence NESHIE susceptibility and outcomes. Unlike NESHIE, for which a limited number of genetic studies have been performed, many studies have identified genetic variants associated with cerebral palsy (CP), which can develop from severe NESHIE. Identifying variants in patients with CP, as a consequence of NESHIE, may provide a starting point for the identification of genetic variants associated with NESHIE outcomes. We have constructed NCGR (NESHIE and CP Genetics Resource), a database of genes and variants reported in patients with NESHIE and CP (where relevant to NESHIE), for the purpose of collating and comparing genetic findings between the two conditions. In this paper we describe the construction and functionality of NCGR. Furthermore, we demonstrate how NCGR can be used to prioritise genes and variants of potential clinical relevance that may underlie a genetic predisposition to NESHIE and contribute to an understanding of its pathogenesis.

1. Introduction

Neonatal encephalopathy (NE) is a heterogenous syndrome of disturbed neurological function in neonates [1]. Hypoxic ischemic encephalopathy (HIE), which results from impaired fetal cerebral blood flow (ischaemia) and oxygen delivery (hypoxia), is a subcategory of NE [2]. HIE is diagnosed based on the presence of clinical and metabolic criteria including low Apgar scores, seizures, metabolic acidaemia and evidence of stroke and neurological damage [1,3]. These diagnostic criteria are not specific to HIE [4–6], and HIE can only be diagnosed once other causes of NE, such as congenital malformations, birth trauma and infection, have been excluded [7]. The multicentre national Neonatal Encephalopathy with Suspected Hypoxic Ischaemic Encephalopathy project in South Africa has chosen to use the term "NE with suspected HIE" or NESHIE.

NESHIE is estimated to account for approximately 35% of neonatal deaths globally [8,9]. The incidence of NESHIE is 1–8 cases per 1000 live births in high-income countries [10] and varies between 2.3 and 26.5 per 1000 live births in low- to middle-income countries (LMIC) [2,8]. For instance, in South Africa, the incidence of NESHIE ranges from 8.5 to 13.3 cases per 1000 live births [6,11–13]. Survivors of NESHIE are at risk of poor neurodevelopmental outcomes and often require lifelong medical care, placing a significant burden on patients and their families [12,14]. The high incidence of NESHIE in LMIC places a burden on limited financial, medical, and social resources [15].

Brain injury in patients with NESHIE may be progressive and occur over a period of time ranging from days to months. The severity of injury involves several cascading processes which occur as a result of the initial hypoxic ischemic event, namely, mitochondrial energy failure, glutaminergic excitotoxicity, inflammation, oxidative stress, and neural cell apoptosis [16,17]. Factors of an environmental nature including maternal age, fetal prematurity, intrapartum cord anomalies and

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maternal fever increase the risk of a fetal hypoxic-ischemic event and subsequent development of NESHIE [18]. However, there is a lack of reliable, sensitive, and specific criteria predicting neurological deterioration in NESHIE patients [19–21]. Environmental factors alone do not explain why some infants with NESHIE have unfavourable responses to therapeutic hypothermia (TH), the current standard of care, while other infants show significant improvement following TH [21,22]. Genetic and epigenetic mechanisms play an important role in the response to hypoxia [23–25], suggesting that a complex interplay between genetic and environmental factors may underlie NESHIE pathogenesis. An underlying genetic predisposition may thus influence outcomes and severity of NESHIE patients. A limited number of studies have attempted to determine whether a genetic predisposition to NESHIE might exist. Thus far only a limited number of genes potentially associated with NESHIE have been identified [26–33]. These include nitric oxide synthase (*NOS*)*3*, which is responsible for the constitutive synthesis of the vasodilator nitric oxide (NO), and the pro-inflammatory cytokines, interleukin (*IL*)*1B* and *IL6* [34,35,36].

Moderate to severe NESHIE can result in the development of cerebral palsy (CP) [37]. CP is defined as a non-progressive set of neurological disorders, characterised by abnormal posture, movement, and muscle tone [38]. The worldwide prevalence of CP is approximately 1–4 cases per 1000 live births [39–47]. NESHIE may account for up to 10–20% of CP cases globally [48–52]. Research has progressed well on the identification of genetic variants underlying a possible genetic predisposition to CP, including CP as a result of NESHIE. Approximately 14–31% of CP cases are suggested to have a genetic cause [53–55]. Candidate genes include collagen type IV alpha 1 chain (*COL4A1*), tubulin alpha 1a (*TUBA1A*) and Adaptor Related Protein Complex 4 Subunit Mu 1 (*AP4M1*) [53]. Since NESHIE is sometimes a causal factor of CP, identifying genes and variants associated with CP that may also influence NESHIE pathogenesis could provide a starting point for the identification of variants that determine moderate to severe NESHIE outcomes. To date, genes involved in inflammatory processes (*IL1B* and *IL6*) and blood flow regulation (the *NOS* family) have been significantly associated with both NESHIE and CP [26,31,56–61]. However, there may be a greater overlap of genes associated with both conditions.

Consolidating genetic data on both CP (where relevant to NESHIE) and NESHIE into a single resource can aid in the identification of novel genes that may be associated with NESHIE. Additionally, as research progresses on whether a genetic predisposition to NESHIE might exist, the importance of collecting data on the genes and variants associated with NESHIE becomes important. With the aforementioned in mind, we have created The NESHIE and CP Genetics Resource (NCGR), a database for collating gene and variant data on NESHIE and CP (where relevant to NESHIE) to enable the comparison of genotypic and phenotypic data between the disorders. NCGR provides the most comprehensive collection to date of data and metadata on genes and short-length variants with potential clinical relevance to NESHIE. Furthermore, NCGR integrates complex search and filter functionality to aid in the comparison and prioritisation of genes and variants that may be involved in a genetic predisposition to NESHIE. Here we describe the construction of NCGR and demonstrate an example of how NCGR can be used to prioritise genes and variants that may influence susceptibility to/severity of NESHIE.

2. Materials and methods

2.1. Construction and content

NCGR (available at: http://ncgr.bi.up.ac.za/) was compiled to provide an overview of publicly-available data on genes and variants with potential clinical relevance, that may underlie a genetic predisposition to NESHIE. NCGR was developed using the Django web-development framework (Django Software Foundation, USA) [62] and Django-based content management system, Wagtail (Torchbox, USA) [63]. Updates to the database will be performed on a biannual basis and researchers are encouraged to contribute their data and suggestions to NCGR.

2.1.1. Inclusion and exclusion criteria

Patients were regarded as having NESHIE if a publication reported (i) diagnosed or suspected HIE based on clinical observation of birth asphyxia/intrapartum hypoxia evidenced by the need for resuscitation shortly after birth, low Apgar scores (≤ 7) and metabolic acidosis (pH $\leq$ 7, base deficit $\geq$ 16 mmol/L), and/or magnetic resonance imaging (MRI)/computerised tomography (CT) findings consistent with hypoxic ischemic injury; and (ii) evidence of NE as determined by one or more neurological manifestations including muscle tone changes, seizures, altered consciousness, tremors or irritability.

To identify variants and their corresponding genes for inclusion in NCGR from the relevant publicly-available scientific literature, a screening approach was adopted as outlined in Fig. 1. Firstly, relevant publications were identified by performing a literature search in the National Centre for Biotechnology Information (NCBI) PubMed database (Fig. 1A). The search terms: (gene AND hypoxic ischemic encephalopathy) OR (variant AND hypoxic ischemic encephalopathy) OR (gene AND cerebral palsy) OR (variant AND cerebral palsy) were used, and articles published between January 2002 and May 2022 were retrieved. Retrieved article titles and abstracts were pre-screened using the following inclusion criteria: (i) condition: patients with NESHIE and/or CP; (ii) language: English; (iii) study type: peer-reviewed case-control, cohort, cross-sectional, case, meta-analyses, genome-wide association, or family-based heritability study; and (iv) study outcome: association of short-length variant [i.e., single nucleotide polymorphism (SNP), splicing site variant or insertion/deletion (INDEL)] in patient/s with the condition. Studies were excluded if they failed to meet the aforementioned inclusion criteria or if at least one of the following exclusion criteria were present: (i) animal-based studies; (ii) studies on known conditions masquerading as NESHIE or CP; and (iii) non-original studies (i.e., reviews, commentaries, editorials, errata, and conference abstracts). Articles meeting the initial pre-screening criteria were subjected to full manual text screening. These articles were further excluded if (i) the criteria above were not met; or (ii) if a CP study had excluded patients with birth asphyxia, HIE or NE from the study population.

Short-length variants from publications that met the above criteria were also subjected to screening according to a tiered approach to determine which variants were suitable for inclusion in NCGR (Fig. 1B). Variants that were reported in patients with NESHIE or CP (as a result of NESHIE/birth asphyxia) formed part of tiers 1 and 2, respectively. These variants and their corresponding genes were catalogued in NCGR if the study reported either (i) statistically significant associations (p-value(s) < 0.05) with the condition; or (ii) predicted pathogenic/likely pathogenic/deleterious effects. The third tier comprised variants reported in patients with CP due to various causation other than that of NESHIE (including idiopathic). Since the cause of CP is often unknown and since diagnosing HIE can be challenging, it is important to compare findings in patients with CP (as a result of NESHIE) to those in CP due to other causes. Tier 3 variants were included if they were located within genes previously catalogued as part of tiers 1 and 2 and if the study reported either (i) statistically significant associations (p-value(s) < 0.05) with CP; or (ii) predicted pathogenic/likely pathogenic/deleterious effects. Variants that were reported as part of a haplotype were not included in NCGR unless the variant was independently associated with NESHIE or CP, since associations between haplotypes and a disease do not specify which variants within a haplotype are causative of the disease.

2.1.2. Data annotation and nomenclature standardisation

Data captured from the publications included the variant and gene coordinates, variant reference and alternate alleles, study population size and demographics, data acquisition methods, and the statistical significance of the study findings. To ensure consistent reporting,

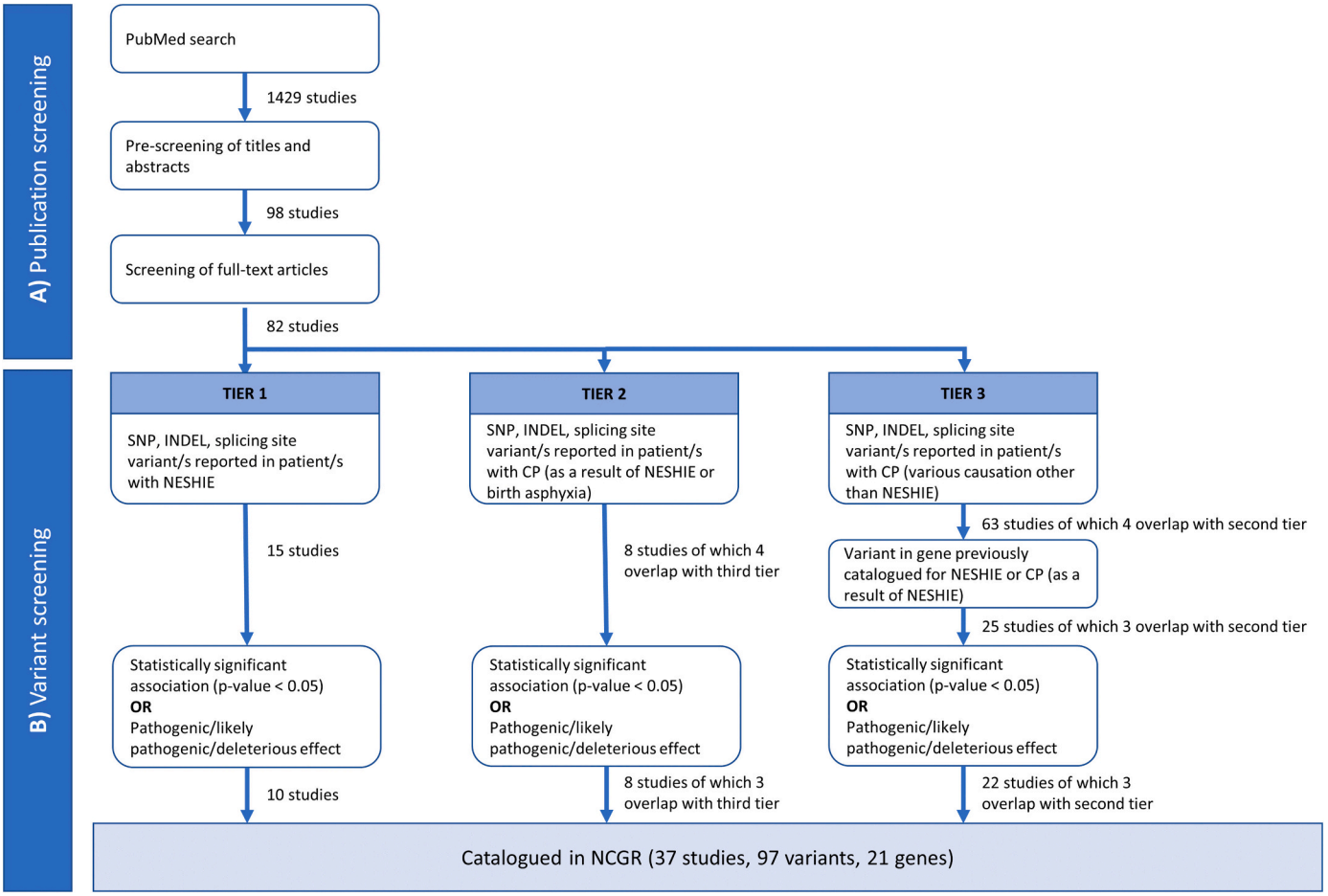

Fig. 1. Screening approach utilised to identify variants and their corresponding genes for inclusion in NCGR. Abbreviations: CP: cerebral palsy; NESHIE: neonatal encephalopathy with suspected hypoxic ischemic encephalopathy; INDEL: insertion and deletion; NCGR: NESHIE and CP genetics resource; SNP: single nucleotide polymorphism.

international standards were used for the naming of variants and genes. Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) official symbols were used to classify genes [64], while variants were classified according to Human Genome Variation Society (HGVS) nomenclature [65] or NCBI Single Nucleotide Polymorphism Database (dbSNP) reference sequence identifiers (RSIDs) [66]. If the HGVS nomenclature for a variant was not provided in a publication, the canonical transcript-based HGVS nomenclature was retrieved using the Ensembl Variant Effect Prediction (VEP) toolset with the reference genome and corresponding genomic coordinates of the variant as input [67]. Additional information from several publicly-available databases was integrated into NCGR to provide researchers with context on the disease-causing potential of the catalogued variants and genes. Variant allele frequencies, variant consequences, and VEP scores were calculated using several algorithms including Functional Analysis through Hidden Markov Models (FATHMM), Sorting Intolerant from Tolerant for genomes (SIFT 4G), Polymorphism Phenotyping v2 (PolyPhen-2) and Combined Annotation Dependent Depletion (CADD), and Ensembl's VEP database. Additionally, MutationTaster VEP scores, which were not available for all variants on Ensembl's VEP database, were retrieved from the MutationTaster database (2021 release) [68]. For catalogued genes, gene Residual Variation Intolerance Scores (RVIS), which provide a measure of genic variation intolerance and consequential disease susceptibility, were included [69]. Additionally, to enable filtering of genes by association with disease phenotypes similar to that of NESHIE or CP, gene-disease relationship information was integrated from the human phenotype ontology (HPO) database [70] using the GeneALaCart GeneCards batch query tool [71]. The HPO database provides an internationally-recognised, unified vocabulary for the description of the clinical features of a disease.

2.1.3. Database design and schema

The PostgreSQL relational database was used to capture study-, variant- and gene-level information which were used as the principal guiding variables of interest during the design of the database structured query language (SQL) schema. The relationship between studies and variants was found to be more accurately described using a graph-theory approach, where the connection between two nodes in a graph may also possess its own unique properties. To represent this, a custom many-to-many through model was used to capture the relationship. The remaining relationships were adequately represented using one-to-many and many-to-many relationships where applicable.

The "Study details" table contains information on NESHIE and CP-related scientific publications, including the publication Digital Object Identifier (DOI) provided by means of a hyperlink to the online publication, study methods and study population size and demographics. By selecting a particular study, users can navigate to the results of that study. Additional information on variants identified in the studies, including the reference genome, genomic location, reference, and alternate alleles, are included in a "Variant details" table. By selecting a variant in this table, users can retrieve a tabulated list of pre-computed variant consequences, effects, and allele frequencies. Lastly, the "Gene details" table provides access to metadata on each candidate gene including the gene symbol, genomic cytoband position, variation

intolerance scores, and associated disease phenotype terms. Selection of a gene in this table provides users with all the disease phenotype terms associated with the gene. Fig. 2 provides a schematic overview of the relational structure of the database.

2.2. Utility

2.2.1. Search and filter functions

Due to the complex nature of biological data and the need for cross-sectional inquiry methods, complex query construction was integrated into NCGR to provide users with advanced data-filtering capabilities. This functionality was achieved using the JQuery QueryBuilder library (https://querybuilder.js.org/), which allows for the creation of complex queries through an intuitive graphical user interface. We utilised QueryBuilder to construct advanced AND/OR expression queries with filters based on disease type, statistical significance, variant consequences, pre-computed variant effect predictions, gene RVIS scores and gene HPO terms. These filters are available on the "Filter and Search" page of NCGR. The code used for construction of the complex filters is available at https://gist.github.com/G-kodes/3103a8bbdeb3c80317313f2ef279c685. In addition to the integration of advanced data filters, all data tables within NCGR were constructed with sortable column headers for categorising results alphabetically and a local search bar whereby users can filter the results of a specified table based on search criteria. If instead a user would like to query the NCGR resource in its entirety for a specific term relating to a gene, variant or its associated metadata, a global search bar accessible at the top of each page can be used.

2.2.2. Demonstration of database functionality

To demonstrate the functionality of NCGR, we performed a complex query to generate a list of candidate genes likely to play a role in predisposing individuals to NESHIE (Supplementary Fig. 1**)**. Genes associated with NESHIE or CP (as a result of NESHIE/birth asphyxia) were selected. These genes were further filtered for those with non-synonymous variants that had (i) statistically significant associations (p-value <0.05); or were (ii) associated with HPO terms similar to that of NESHIE, namely, "cerebral ischemia" or "tissue ischemia". Known disease genes often cause diseases with similar phenotypes, thus by selecting for genes associated with HPO terms similar to that of NESHIE, we can identify genes that may not previously have had significant associations with NESHIE but may make good candidates for future genetic studies.

The final list of prioritised genes was analysed for their potential roles in NESHIE pathogenesis by (i) identifying enriched gene ontology (GO) terms; and (ii) examining the interactions between the gene-encoded proteins. To identify recurring processes, a GO analysis of the input list of genes was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resource [72,73]. Enriched biological process (BP), molecular function (MF) and cellular component (CC) ontologies were examined. The Search Tool for Retrieval of Interacting Genes (STRING) database tool v11.5 was utilised to construct a protein-protein interaction network using the list of prioritised genes as input [74]. To examine the interactions of our prioritised proteins with neighbouring proteins, a maximum of 10 additional directly-interacting proteins was included in the first shell of the network, and a maximum of 50 indirectly-interacting proteins was included in the second shell. The network was visualised using Cytoscape software v3.9.0 [75]. Additional details on the methods used to perform the process/pathway enrichment analysis and construct the protein-protein interaction network and are provided in **Supplementary Data A**.

3. Results

3.1. Database overview

An initial PubMed search yielded 1429 articles of potential relevance for inclusion in NCGR. Of these articles, 1331 were excluded on the basis of title and/or abstract content. The full content of the remaining articles was manually screened, and an additional 16 articles were excluded. Notably, a letter to the editor by Li et al. which stated a significant association of matrix metallopeptidase 3 (*MMP3*) rs2070744 with NESHIE was excluded at this stage as the authors did not provide information on the diagnostic criteria for NESHIE [76]. Of the 82 remaining articles, 37 contained variants of relevance according to specified criteria and were included in NCGR, along with their corresponding genes (Fig. 1). NCGR therefore contains data and metadata on 21 candidate genes and 97 variants retrieved from 37 peer-reviewed studies. Of these studies, 10 reported on variants in NESHIE patients and 27 on variants in CP patients including those with CP as a result of NESHIE/birth asphyxia. All gene and variant data and metadata utilised in the construction of NCGR are provided in Supplementary Tables 1-7.

3.1.1. Study methodology

NESHIE-specific studies catalogued in NCGR predominantly used polymerase chain reaction (PCR)-based methods for the detection of variants. High-throughput and high-coverage whole genome sequencing, whole exome sequencing and microarray methods have not been published to date; therefore, sections of the genome still remain unexplored for variants that might predispose to NESHIE. Although high-throughput and high-coverage techniques have been used to identify variants associated with CP, many of the preliminary studies have been statistically underpowered [53]. Additionally, inconsistencies in the naming of genes and variants in several earlier CP studies have prevented cross-referencing of variants between studies. We recommend that consistent international naming conventions, such as those established by the HGVS, are implemented for future genetic studies on NESHIE and CP to ensure traceability of variants.

3.1.2. Population demographics

Study populations in NCGR were classified by geographic region. The most represented study population region was East Asia ($n = 18$), followed by Europe ($n = 13$). In comparison, other population regions including South Asia ($n = 2$), and Africa ($n = 4$), were underrepresented. Of the studies with populations classified under African descent, two included data from individuals of African-American ancestry [60,77], one from individuals of unspecified African ancestry [78], and one from individuals of North African (Sudan) and sub-Saharan African (Somali) ancestry [79,80]. Other than the latter study, which investigated whether an underlying genetic predisposition exists in individuals with atypical CP, no other genetic studies on NESHIE or CP have been performed to date using data from populations of recent African origin.

3.1.3. Replicated findings

All catalogued genes, with the exception of oligodendrocyte transcription factor 2 (*OLIG2)* [32], adapter related protein complex 4 subunit beta 1 (*AP4B1)* [81] and pantothenate kinase 2 (*PANK2*) [32,82] were reported in at least two NESHIE and/or CP studies. Recurrent variants (catalogued in at least two studies) were caspase recruitment domain family member 8 (*CARD8*) rs2043211 [31,83,84], catalase (*CAT*) rs1001179 [31,84], eukaryotic translation initiation factor 4E family member 2 (*EIF4E2*) c.214C > G [55,85], G protein subunit alpha O1 (*GNAO1*) c.625C > T [77,86], *IL1B* rs16944 [31,56,57,83], *IL6* rs1800795 [26,60,78,87] and rs2069837 [58,59], *IL10* rs1800896 [57,88], methylenetetrahydrofolate reductase (*MTHFR*) rs1801131 [89,90] and rs1801133 [90–93], and *NOS3* rs1800779 [92,94] and rs1808593 [27,28]. Two of the recurring variants, namely, *CAT* rs1001179 and *IL1B* rs16944 were significantly associated ($p <$

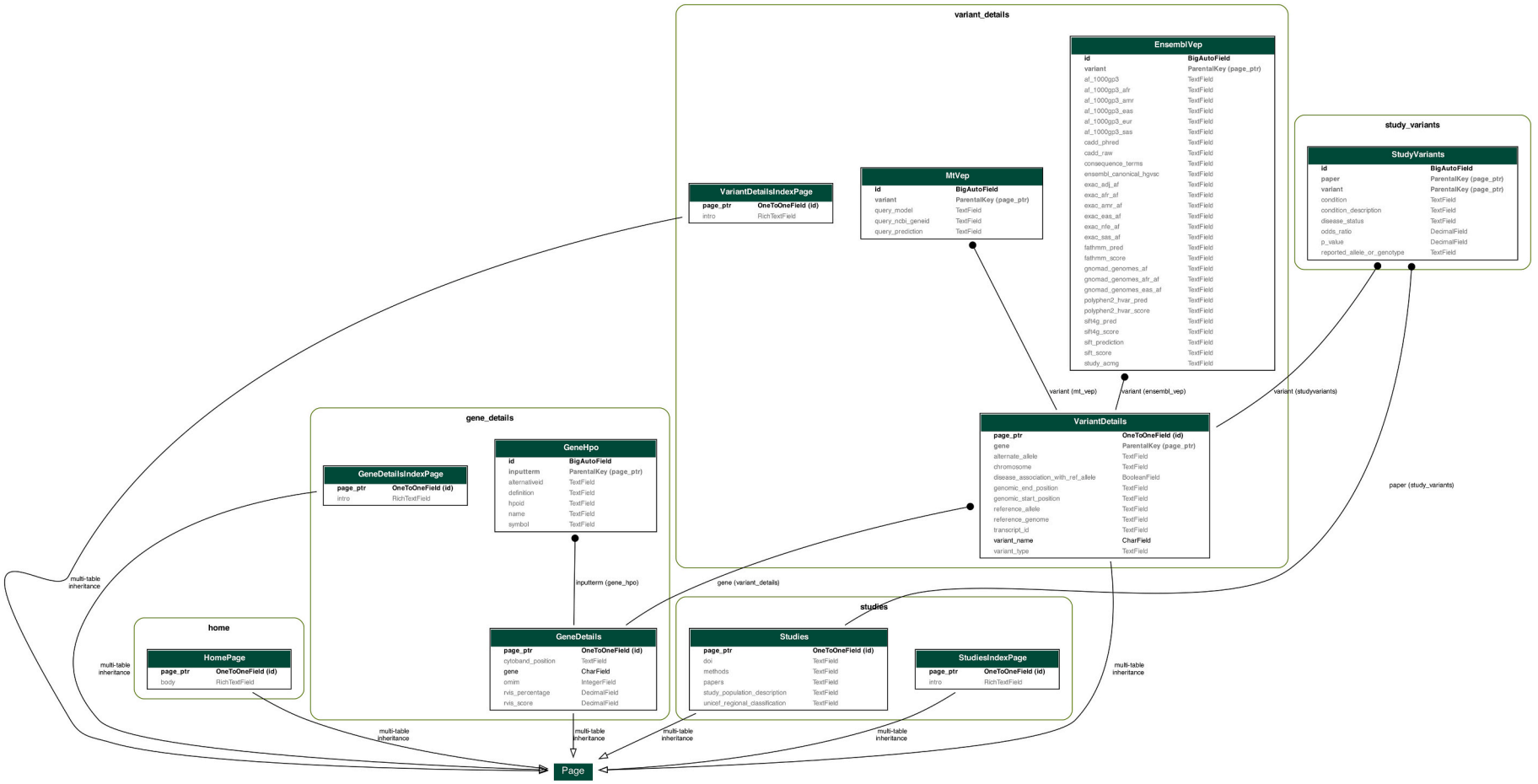

Fig. 2. Database schema and information storage visualised as an entity-relation diagram.

0.05) with both NESHIE and CP (as a result of NESHIE), while *IL6* rs1800795, *IL10* rs1800896 and *MTHFR* rs1801133 were significantly associated with NESHIE and CP (various aetiologies other than NESHIE). While none of the *COL4A1* variants were reported more than once, a high number of variants in *COL4A1* with predicted deleterious/pathogenic effects were reported in CP patients across several studies [77,85,86,95,96]. One of the catalogued *COL4A1* variants with predicted deleteriousness, NM_001845:c.2263G > A, occurred in a patient with CP due to birth asphyxia, which describes a hypoxic event that may lead to NESHIE and potentially to CP [82,97]. In addition to variants in COL4A1, variants in another gene that forms part of the COL4 family namely, COL4A4, have also been reported in patients with CP [96,98]. Notably, the COL4A4 stop-gain variant, NM_000092.5:c.4720C > T, was reported in a patient with CP as a result of NESHIE [96].

3.2. Database functionality

3.2.1. Gene prioritisation

To demonstrate the type of output that can be obtained from NCGR, the search and filter features were utilised to prioritise candidate genes and variants that were likely be associated with NESHIE. In total, 12 genes met the prioritisation criteria. Of the prioritised genes, the angiotensinogen (*AGT*), *AP4B1*, *CARD8*, *CAT*, *IL6*, *IL10*, *IL1B*, *MTHFR*, *NOS3*, *OLIG2* and *TNF* genes had statistically significant associations with NESHIE or CP (as result of NESHIE). Although variants in *COL4A1* have not been significantly associated with NESHIE or CP as a consequence of NESHIE, the *COL4A1* gene was prioritised since (i) the gene has prior associations with cerebral ischemia and tissue ischemia disease phenotypes; and (ii) variants in the gene have been reported in patients with CP (as a result of birth asphyxia) as previously mentioned [82]. Table 1 provides additional details on the prioritised genes, including brief functional descriptions and associated HPO terms.

3.2.2. Gene ontology and protein-protein interactions

GO enrichment analysis of the input list of prioritised genes identified a statistically significant (Benjamini-corrected p-value $\leq$ 0.05) enrichment of genes involved in biological processes of inflammation (GO:0006954, GO:0050729, GO:0001819), and the closely related processes of angiogenesis through vascular endothelial growth factor (VEGF) production, and vasodilatation (GO:0010573, GO:0042311, GO:0045429). Notably, there was a high enrichment of genes involved in the negative regulation of neurogenesis (GO:0050768; n = 3, corrected p-value = 0.0067) and the positive regulation of nuclear factor-kappa-beta (NFKB) signalling (GO:0043123; n = 4, corrected p-value = 0.0067), which is responsible for initiating multiple innate and adaptive immune responses through regulation of cell proliferation, differentiation, apoptosis, and angiogenesis [99]. Interestingly, TNF, MTHFR and CAT were also involved in the response to hypoxia (GO:0001666) albeit at a non-significant level. The generated GO enrichment results are provided in **Supplementary Data B**.

A protein-protein interaction network was constructed using the STRING database to briefly examine the physical and functional interactions between the protein products of the prioritised genes and their interacting proteins. The network consisted of 72 nodes and 363 edges of which 70 nodes formed the main connected component (Supplementary Fig. 2). The NESHIE-associated proteins, IL6, IL1B, and TNF, were direct interactors within the network and were significantly enriched in GO inflammatory processes including the cytokine-mediated signalling pathway (GO:0019221, GO:0005125). TNF, a pro-inflammatory cytokine, had the highest degree of connectivity in comparison to other proteins in the network with 28 neighbouring proteins. These neighbouring proteins included those involved in the aforementioned inflammatory cytokine processes (IL1B and IL6), the anti-inflammatory cytokine, IL10, and caspase 8 (CASP8) and caveolin 1 (CAV1), which are direct interactors of COL4A1 and NOS3, respectively.

4. Discussion

Recent studies suggest that a complex interplay of both environmental and genetic factors may influence NESHIE susceptibility and outcomes [25–31,33]. Moderate to severe NESHIE often results in CP [37], which is well-studied in terms of genetic susceptibility/predisposition. Identifying genetic variants underlying CP (as a result of NESHIE) may provide a starting point for identifying genetic variants that may predispose to NESHIE. To date, no resource exists collating genetic data on both NESHIE and CP (where relevant to NESHIE) to enable a comparison of findings between the conditions. Additionally, as the number of studies investigating the existence of a genetic predisposition to NESHIE increases, there is a need for a centralised database for the storage of genetic findings associated with this condition. With the aforementioned in mind, we have constructed NCGR, a resource for the collation and comparison of genes and variants reported in patients with NESHIE and CP (where relevant to NESHIE).

The findings of several NESHIE and CP studies catalogued in NCGR were replicated. Notably, the *MTHFR* rs1801133 GA/AA genotype, which is known to cause elevated plasma homocysteine levels [100,101], was significantly associated with white matter/watershed (WM/WS) brain injury patterns in full-term infants with NESHIE [91]. WM/WS brain injury patterns are typically observed in full-term NESHIE patients with less severe injury caused by partial prolonged asphyxia [102,103], although injury to these brain areas may still result in CP [104]. In addition to NESHIE infants with mild-moderate injury, the rs1801133 A allele has also been associated with mothers of NESHIE patients with cystic encephalomalacia, a brain injury pattern indicative of severe brain injury [93,105]. Since moderate to severe NESHIE can lead to the development of CP, it may be unsurprising that rs1801133 has also been associated with CP. Gibson and colleagues reported that the less frequent GA/AA genotype significantly increases susceptibility to CP in girls and premature infants (32–36 weeks gestation), and the risk of development of diplegic CP [90,92]. In contrast, the same group reported that the reference GG genotype is protective for quadriplegic CP [90]. Despite independent associations of rs1801133 with NESHIE and CP, the association of this variant with CP (as a result of NESHIE) has not yet been reported.

Additional, notable variants replicated in the studies were *IL1B* rs16944 and *CAT* rs1001179. *IL1B* rs16944 was significantly associated with periventricular leukomalacia (PVL) and posterior limb of the internal capsule (PLIC) brain damage in full-term NESHIE patients [31,57]. PVL is a known indicator of mild to moderate NESHIE in preterm neonates and children with PVL are known to develop spastic forms of CP [102]. *IL1B* rs16944 was also significantly associated with an increased risk of CP in NESHIE patients and epilepsy in NESHIE patients that were also carriers of the *CARD8* rs2043211 allele [56,83], suggesting a role for this variant in NESHIE outcomes both independently or through interactions with other variants. Like that of *IL1B* rs16944, the *CAT* rs1001179 variant was also associated with both NESHIE and CP (as a result of NESHIE) [31,84]. However, the rs1001179 T allele was associated with susceptibility to CP (as a result of NESHIE) in a 2016 study by Esih and colleagues but was later reported as being protective against severe brain damage in NESHIE by the same group [31,84]. Esih and colleagues hypothesised that these inconsistencies may be caused by differences in the treatment regimens used for patients in the two groups. Additional studies are needed to confirm this.

To demonstrate the search and filter functionality of NCGR, we performed a complex query of the database to prioritise candidate genes of potential clinical relevance. In total, 12 genes were identified of which 11 had a statistically significant association with NESHIE and/or CP as a result of NESHIE. Of these, *IL1B*, *NOS3* and *TNF* were reported in at least three studies catalogued in NCGR and had negative RVIS scores indicative of intolerance to variation [27,28,31,33,56,57,83,92,106–109]. Genes with an intolerance to variation show high levels of evolutionary conservation and are thus most likely to incorporate

Table 1
Candidate genes that may underlie a genetic predisposition to NESHIE.

Gene	Associated condition	Reproducibility (Identified in > 1 study in NCGR)	Intolerant to variation (RVIS < 0)	Statistically significant association (p-value < 0.05) with NESHIE or CP (as a result of NESHIE)	Protein Function	Relevant HPO terms	Reference
AGT	NESHIE	No	No	Yes	Regulation of blood pressure	Abnormal cardiovascular system physiology; Abnormality of the respiratory system; Hypotension	[29]
AP4B1	CP (as a result of NESHIE)	No	Yes	Yes	Vesicle-mediated transport of proteins	Abnormal nervous system physiology	[81]
CARD8	NESHIE	Yes	No	Yes	Regulation of apoptosis and upregulation of inflammation through activation of NFKB	Abnormality of immune system physiology; Abnormal inflammatory response	[31,83]
CAT	NESHIE and CP (as a result of NESHIE)	Yes	Yes	Yes	Antioxidant enzyme responsible for the breakdown of hydrogen peroxide into water	Abnormality of the cardiovascular system morphology; Abnormal vascular morphology; Abnormality of the respiratory system; Abnormality of immune system physiology; Abnormal inflammatory response	[31,84]
COL4A1	CP (as a result of birth asphyxia and various aetiologies other than NESHIE)	Yes	Yes	No	Main component of the vascular basement membrane; role in the maintenance of vascular tone	Abnormal cardiovascular system physiology; Abnormal cardiovascular system morphology; Abnormality of blood circulation; Abnormality of the vasculature; Vascular dilatation; Tissue ischemia; Cerebral ischemia	[77,82,85,86,95,96,97,132]
IL10	NESHIE and CP (various aetiologies other than NESHIE)	Yes	No	Yes	Suppression of inflammation	Abnormal cardiovascular system physiology; Abnormal vascular physiology; Abnormal respiratory system physiology; Abnormal inflammatory response; Tissue ischemia; Cerebral ischemia	[57,88]
IL1B	NESHIE and CP (as a result of NESHIE)	Yes	Yes	Yes	Upregulation of inflammation		[31,56,57,83]
IL6	NESHIE and CP (various aetiologies other than NESHIE)	Yes	No	Yes	Both the suppression and upregulation of inflammation	Abnormality of cardiovascular system morphology; Abnormal inflammatory response	[26,58,59,60,78,87]
MTHFR	NESHIE and CP (various aetiologies other than NESHIE)	Yes	No	Yes	Enzyme responsible for the processing of the amino acid, homocysteine	Abnormality of the cardiovascular system physiology; Abnormal vascular physiology; Abnormal cerebral vascular morphology	[89,90,91,92,93,107]
NOS3	NESHIE	Yes	Yes	Yes	Endothelial vasodilator; mediator of angiogenesis	Abnormal cardiovascular system physiology; Abnormal nervous system physiology; Abnormality of the vasculature; Abnormal systemic blood pressure; Increased blood pressure	[27,28,33,92,94]
OLIG2	CP (as a result of NESHIE)	No	No	Yes	Differentiation of motor neuron and oligodendrocytes		[32]
TNF	NESHIE and CP (various aetiologies other than NESHIE)	Yes	Yes	Yes	Upregulation of inflammation; apoptosis; angiogenesis	Abnormality of the nervous system; Abnormality of immune system physiology; Abnormal respiratory system physiology	[57,92,107,108,109]

pathogenic, disease causing variants [110]. *COL4A1* did not have significant associations with NESHIE but was prioritised on the basis of an association with cerebral and tissue ischemia. *COL4A1* provides the instruction for producing type IV collagen, an essential component of the vascular basement membrane. It is also among the top 0.62% of variation intolerant genes (RVIS score = −2.82) and has been associated with several notable phenotypes other than that of ischemia including vascular abnormalities, seizures and encephalopathy [111–114]. Thus, *COL4A1* could provide a strong candidate for future studies investigating the existence of a genetic predisposition to NESHIE. Additional studies are necessary to elucidate whether variants in *COL4A1* are associated with NESHIE.

Biological processes and pathways associated with the input list of prioritised genes were retrieved by performing GO and pathway enrichment analysis of this network. Significantly enriched biological processes associated with the protein products of our prioritised genes include vasodilatation (*TNF*, *AGT* and *NOS3*), and inflammation (*IL1B*, *IL6*, *IL10*, *TNF* and *CARD8*). Vasodilatation and the closely-related process of angiogenesis are initiated as protective mechanisms after a hypoxic-ischemic event and are important for the restoration of blood flow and oxygen to tissues affected by hypoxia [115]. In contrast, inflammation, which is triggered by induction of the immune system after hypoxia-ischemia, is a leading cause of brain injury in neonates with NESHIE [17]. It is plausible that variants causing reduced or altered function in genes involved in the restoration of blood flow, such as *NOS3* rs1800783 which has been proposed to reduce gene expression of the NOS3 vasodilator [27], or contributing to inflammation may impact NESHIE outcomes and contribute to the risk of developing CP. In support of the latter, the NESHIE- and CP-associated variants, *IL1B* rs16944 and *IL6* rs1800795 catalogued in NCGR are associated with increased expression of their respective pro-inflammatory cytokines, IL1B and IL6 [56,116–120]. These cytokines have been associated with inflammation and adverse clinical outcomes in NESHIE patients [121–123]. Thus, variants in *IL1B* and *IL6* may underlie a genetic basis for inflammation in some patients with severe NESHIE.

The NFKB signalling pathway was also enriched within our gene list. In addition to previously discussed roles in inflammation and vasodilatation, *TNF*, *IL1B* and *AGT* also have important roles in the mediation of apoptosis through involvement in the NFKB pathway, among others. The NFKB pathway regulates several immunological processes including the induction of inflammation by expression of pro-inflammatory genes, the stimulation of apoptosis in response to stress, and the induction of angiogenesis [124]. Interestingly, the NFKB pathway is activated in response to hypoxia, whereby it initiates expression of pro-inflammatory genes, decreased apoptosis and increased angiogenesis [125]. The NFKB pathway has been shown to have a dual role in the pathogenesis of NESHIE by contributing both to brain damage through induction of inflammation and neuroprotection through suppression of apoptosis [126]. Although not on our list of prioritised genes, genes central to the NFKB pathway, such as *NFKB1*, may also make good targets in the future for studies investigating whether a genetic predisposition to NESHIE exists.

One of the most crucial limitations of the research done to date is an under-representation of variant data from certain population groups. The catalogued studies in NCGR have predominantly used sequencing data of East Asian and European ancestry. Data from several population groups including Africans is underrepresented. It is imperative to perform research using genetic data from individuals from the African continent, specifically due to the high genetic diversity in African populations [127]. The high genetic diversity results in many haplotypes (combinations of alleles) and consequently shorter haploblocks (regions with little evidence of historical genetic recombination) in African genomic sequences [127,128]. Thus, the frequencies of variant alleles in African populations are vastly different from those seen in other populations, with rare alleles (allele frequency < 0.5%) occurring more often [129]. Rare variants have larger effect sizes and are likely to decrease reproductive fitness and contribute to disease [129–131]. Therefore, studying African population data may provide even greater insight into genetic variants contributing to NESHIE and CP pathogenesis.

5. Conclusion and future directions

To our knowledge, NCGR provides the largest compilation to date of publicly-accessible information on variants and genes reported in patients with NESHIE and CP (where relevant to NESHIE). Utilisation of NCGR allows researchers to identify patterns and gaps within research to date, including infrequently analysed population-groups, under-utilised study methodology, as well as replicable variants and genes. Furthermore, access to additional metadata on genes and variants, including gene-disease phenotype association data, genetic intolerance scores, and precomputed variant consequences and effect predictions, may aid researchers in the investigation of potential disease-causing variants and genes catalogued in NCGR. Through utilisation of filter and search features built into NCGR, researchers can prioritise genes and variants specific to their research purposes.

At present, NCGR houses data and metadata on genes and short-length variants of potential clinical relevance. In the future, to enable a comparison of all findings, it may be important to expand the scope of the database to include all reported variants, including CNVs and haplotypes. To aid future research, it may also be important to include additional information on the study methods of articles catalogued in NCGR, such as potential confounding factors or methodology limitations that may influence association results. Additionally, as genetic studies on NESHIE and CP increase, NCGR may become more difficult to update and maintain. To counteract this challenge, new methods which utilise machine-learning and other computational approaches for the entry of data, will need to be implemented.

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Ethics approval

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CRediT authorship contribution statement

Megan A. Holborn: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. **Graeme Ford:** Formal analysis, Methodology, Software, Writing – original draft. **Sarah Turner:** Data curation, Formal analysis, Writing – original draft. **Juanita Mellet:** Writing – review & editing, Supervision. **Jeanne van Rensburg:** Conceptualization, Writing – review & editing, Resources. **Fourie Joubert:** Writing – review & editing, Supervision. **Michael S. Pepper:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors have no competing interests to declare.

Data availability

All data generated or analysed during this study are included in this published article, supplementary information files, and the NCGR database repository. Access to the NCGR database is available at http://ncgr.bi.up.ac.za/. Additional data will be made available on request.

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