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Phenotypic Divergence of *JAG1*- and *NOTCH2*-Associated Alagille Syndrome & Disease-Specific *NOTCH2* Variant Classification Guidelines

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Received: 3 February 2025 | **Revised:** 30 May 2025 | **Accepted:** 16 July 2025

Handling Editor: Luca Valenti

Funding: We would like to thank the following agencies for their generous funding support: The Alagille Syndrome Alliance, Mirum Pharmaceuticals Inc. and Ipsen, who provided unrestricted educational grants to the Hospital for Sick Children (SickKids Foundation). MAG and NBS are supported by the NIH (R01DK134585 and R01DK140468) and the Evelyn Willing Bromley Chair in Paediatric Pathology at The Children's Hospital of Philadelphia. The National Natural Science Foundation of China (81873543 and 81570468) provided funding to the Children's Hospital of Fudan University, The Centre for Paediatric Liver Diseases, Shanghai, China.

Keywords: Alagille syndrome | cholestasis | genetics | NOTCH2

ABSTRACT

Background & Aims: Alagille syndrome (ALGS) is a rare, autosomal dominant disorder with high phenotypic heterogeneity. Disease-causing variants are primarily identified in Jagged1 (*JAG1*), with fewer reported in *NOTCH2*. *JAG1* variants cause disease through a mechanism of haploinsufficiency, but the mechanism for *NOTCH2* variants is not completely understood, making classification of variants more challenging. Using a large, international patient cohort acquired through the Global ALagille Alliance (GALA) study, we sought to improve classification of *NOTCH2* variants and study phenotypic differences between *NOTCH2*- and *JAG1*-related disease.

Melissa A. Gilbert and Binita M. Kamath contributed equally to this study.

For affiliations refer to page 12.

Methods: Clinical and molecular data from 952 individuals with ALGS in GALA were analysed and disease features compared between those with *JAG1* ($n = 902$) and *NOTCH2* ($n = 34$) variants. Previously reported and newly identified *NOTCH2* variants were reinterpreted based on disease-specific modifications to the American College of Medical Genetics and Genomics (ACMG) guidelines. The Kaplan–Meier method was utilised to assess native liver survival (NLS) and overall survival (OS) and gene comparisons were made with the log-rank test.

Results: Thirty *NOTCH2* variants, including 18 novel variants, were identified and classified in our GALA cohort. Phenotypic analyses revealed a significantly lower incidence of characteristic facies, posterior embryotoxon, cardiac involvement and butterfly vertebrae in individuals with *NOTCH2* variants compared to those with *JAG1* variants ($p < 0.001$). No differences were identified in NLS or OS. Review of 61 previously reported *NOTCH2* variants resulted in the re-classification of 19 likely pathogenic or pathogenic to VOUS (31.1%) with less than half retaining their originally published classification (34.4%; $n = 21$).

Conclusions: We report on a large global study on *NOTCH2* genetics and phenotype, which increases the number of reported *NOTCH2* variants by 30%. All variants were reclassified using current guidelines, and comparison of the *JAG1* and *NOTCH2* cohorts demonstrates clear phenotypic divergence between these groups. These data suggest that reliance on classical clinical phenotyping may miss patients with *NOTCH2*-related disease and supports an inclusive approach to genetic testing.

1 | Introduction

Alagille syndrome (ALGS) is an autosomal dominant, multisystem disorder that is the most common inherited cause of neonatal cholestasis, with an overall incidence of 1:30 000 [1]. Additional clinical features include characteristic facies, cardiac, skeletal, renal, vascular and ocular involvement [2–6]. The molecular aetiology of ALGS stems from dysfunctional Notch signalling caused by pathogenic variants in either the Notch pathway ligand Jagged1 (*JAG1*) or the Notch receptor, *NOTCH2*, which account for 94.3% and 2.5% of cases, respectively [7]. A clinical diagnosis of ALGS relies on the presence of at least three disease features or the presence of one disease feature and either a family history in a first degree relative or a confirmed pathogenic/likely pathogenic variant identified in *JAG1* or *NOTCH2*.

JAG1-related ALGS has been well-characterised with over 700 variants described in the Human Gene Mutation Database (HGMD) [8]. The majority of *JAG1* variants (including full gene deletions) result in loss-of-function (LoF) of the JAG1 protein, implicating haploinsufficiency as the underlying disease mechanism [4, 9]. In ALGS, there is remarkable variability in both disease severity and organ involvement including among family members harbouring the same pathogenic variant [3, 10–14]. The mechanisms underlying variable expressivity remain unknown but likely involve the contribution of genetic modifiers [15–18]. Consequently, cohort-based studies have failed to establish a genotype–phenotype association among patients with ALGS [19–21].

The functional consequences of variants in *NOTCH2* are less well understood, with only 35 variants reported in HGMD [8]. Given the paucity of supportive functional data and the low number of individuals with a *NOTCH2* variant, variant of uncertain significance (VOUS) rates for *NOTCH2* are high. A recent study reporting sequencing results from a cholestatic gene panel published a VOUS rate of 91.7% for *NOTCH2* in a large cohort of patients with cholestasis [1]. This uncertainty is reduced within cohorts meeting clinical diagnostic guidelines for ALGS (64% in ClinVar, a database of DNA variants and their associated phenotypes), but remains substantial [22]. Phenotypic differences between *NOTCH2*- and *JAG1*-related ALGS have been noted, including a reduced incidence of cardiac, skeletal and facial features, although these findings were drawn from a small cohort of only eight individuals with *NOTCH2* variants, inhibiting definitive conclusions [23]. *NOTCH2* variants have also been shown to be a cause of Hajdu-Cheney syndrome, which includes a spectrum of disorders, such as Serpentine fibula-polycystic kidney syndrome, that primarily affect skeletal formation, among other features. Variants associated with Hajdu-Cheney and related syndromes are distinct in both their location within *NOTCH2*, with all occurring within a specific region in the last exon of the gene, and pathomechanism (gain-of-function) [24, 25].

The Global ALagille Alliance (GALA) study is an international initiative aimed to chronicle clinical and genetic data from individuals with ALGS. We have curated a large and geographically diverse cohort of 952 individuals, allowing us to reclassify previously reported *NOTCH2* and *JAG1* variants, offering disease-specific variant interpretation guidelines. This study presents the largest *NOTCH2* cohort described to date. Additionally, we carried out deep clinical phenotyping and genotype interpretation to identify phenotypic differences between *JAG1*- and *NOTCH2*-associated ALGS.

2 | Patients and Methods

2.1 | GALA Patient Cohort

The GALA Study Group was established in 2018 and consists of 89 medical institutions from 35 countries [26]. The study protocol and its implementation across participating global centres is described in detail elsewhere [26]. This observational cohort study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [27]. For this analysis, we ascertained individuals who underwent genetic testing for *JAG1* and/or *NOTCH2* (Table S1). Genetic testing strategies varied by institution, but could include: *JAG1* and *NOTCH2* single gene or panel sequencing and deletion/duplication analysis,

Summary

- We studied a large, international cohort of individuals with Alagille syndrome (ALGS) and describe the largest group of patients with changes in the gene *NOTCH2* described to date.
- Comparison of individuals with *NOTCH2* variants to individuals with the more commonly identified *JAG1* variants showed clear differences in how the disorder manifests.
- This suggests that relying only on typical signs and symptoms may miss cases of ALGS due to *NOTCH2* variants and supports broader genetic testing in individuals who do not meet the classic clinical phenotype.

exome sequencing and genome sequencing. The study was approved by the ethics committee at each participating centre or an exemption from ethics approval was granted in accordance with institutional regulations.

2.2 | Classification of NOTCH2 and Missense JAG1 Variants

American College of Medical Genetics and Genomics (ACMG) guidelines were used to classify all *NOTCH2* variants and all missense *JAG1* variants identified within the GALA cohort, as well as all *NOTCH2* variants previously reported in the literature [28]. These guidelines provide a list of criteria that can be used as evidence to support either benignity or pathogenicity. Each criterion is given a specified weight, with some providing stronger support of pathogenicity than others, and the collective evidence for a variant is used to guide classification. Modifications of these guidelines were applied based on updated recommendations and our expertise in ALGS genetics (Table S2) [29]. Minor allele frequency (MAF) cut-offs for both *JAG1* (3.33E-05) and *NOTCH2* (8.33E-07) were established based on the frequency of causative variants identified in each gene and were used to guide utilisation of population databases (gnomAD v.2.2.1 and v.3.1.2).

2.3 | Curation of Previously Reported NOTCH2 Variants (External to GALA)

Previously reported *NOTCH2* variants were identified from the Human Gene Mutation Database (HGMD) (v.2024.3) where variants were filtered to include only those that were reported to be disease-causing (DM) or likely disease-causing (DM?) and that were associated with ALGS [8]. *NOTCH2* variants were also identified from ClinVar (last queried on March 11, 2024) and were filtered to include only those reported as 'pathogenic' or 'likely pathogenic' and that listed 'Alagille syndrome' as the associated condition [22]. Additionally, a literature search on PubMed for *NOTCH2* was performed with a last check on 11 March 2024. Results from all queries were reviewed, and *NOTCH2* variants were excluded if (1) variants were reported with bi-allelic inheritance, (2) variants did not segregate in affected individuals, (3) variants were identified in individuals in whom a pathogenic variant in *JAG1* was also detected and (4) protein-truncating variants were identified in the PEST domain (associated with Hajdu-Cheney syndrome) [25].

2.4 | Statistical Analysis

Summary statistics are presented using medians and interquartile ranges (IQR), and categorical variables are reported as counts and percentages. Demographic and clinical characteristics were compared between genotype groups using the Chi-square test or Fisher's exact test, as appropriate. Native liver survival (NLS) and overall survival (OS) were calculated utilising the Kaplan–Meier method, with group comparisons carried out using the log-rank test. Data were censored at the last known follow-up, upon reaching 18 years of age or on 31 August 2019, whichever occurred first.

To investigate genotype–phenotype correlations in ALGS-related genes, individuals harbouring pathogenic/likely pathogenic or VOUS in *NOTCH2* were compared to those with a *JAG1* pathogenic/likely pathogenic or VOUS. For *NOTCH2*, individuals were further divided into three groups: (1) protein-truncating (frameshift and nonsense), (2) splice site and (3) non-protein-truncating (missense) for intergenotype comparisons. Similarly, for *JAG1*, individuals were further stratified into four groups: (1) protein-truncating (frameshift and nonsense), (2) splice site, (3) non-protein-truncating (missense and in-frame deletions) and (4) structural (full gene deletions, single or multi-exon deletions, multi-exon or full-gene duplications and translocations) for additional group comparisons. A series of sensitivity analyses were conducted, excluding individuals with VOUS in both ALGS disease genes (*JAG1* and *NOTCH2*) to assess the robustness of the primary findings. A p-value < 0.05 was considered statistically significant, and the analysis was performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL) version 25.

3 | Results

3.1 | GALA Patient Cohort

At the time of data extraction, a total of 1543 participants with ALGS were reported in the GALA database. Of these participants, 591 did not meet study requirements and were excluded from further analysis. The majority of exclusions were attributed to a lack of genetic testing ($n = 343$), missing or incomplete variant details ($n = 197$), or incomplete genetic testing ($n = 51$). The final cohort consisted of 952 participants (56.6% male) from 66 centres in 29 countries (Figure 1).

The majority of study participants were probands (95.7%, $n = 912/952$). A pathogenic/likely pathogenic or VOUS in *JAG1* or *NOTCH2* was identified in 98.3% ($n = 936/952$) of participants, with no variant identified in either gene for 1.7% ($n = 16/952$). The majority of individuals were identified to have a variant in *JAG1* (94.7%, $n = 902$), with a minority of patients reporting a finding in *NOTCH2* (3.6%, $n = 34$). Table 1 summarises the clinical characteristics of the entire study cohort.

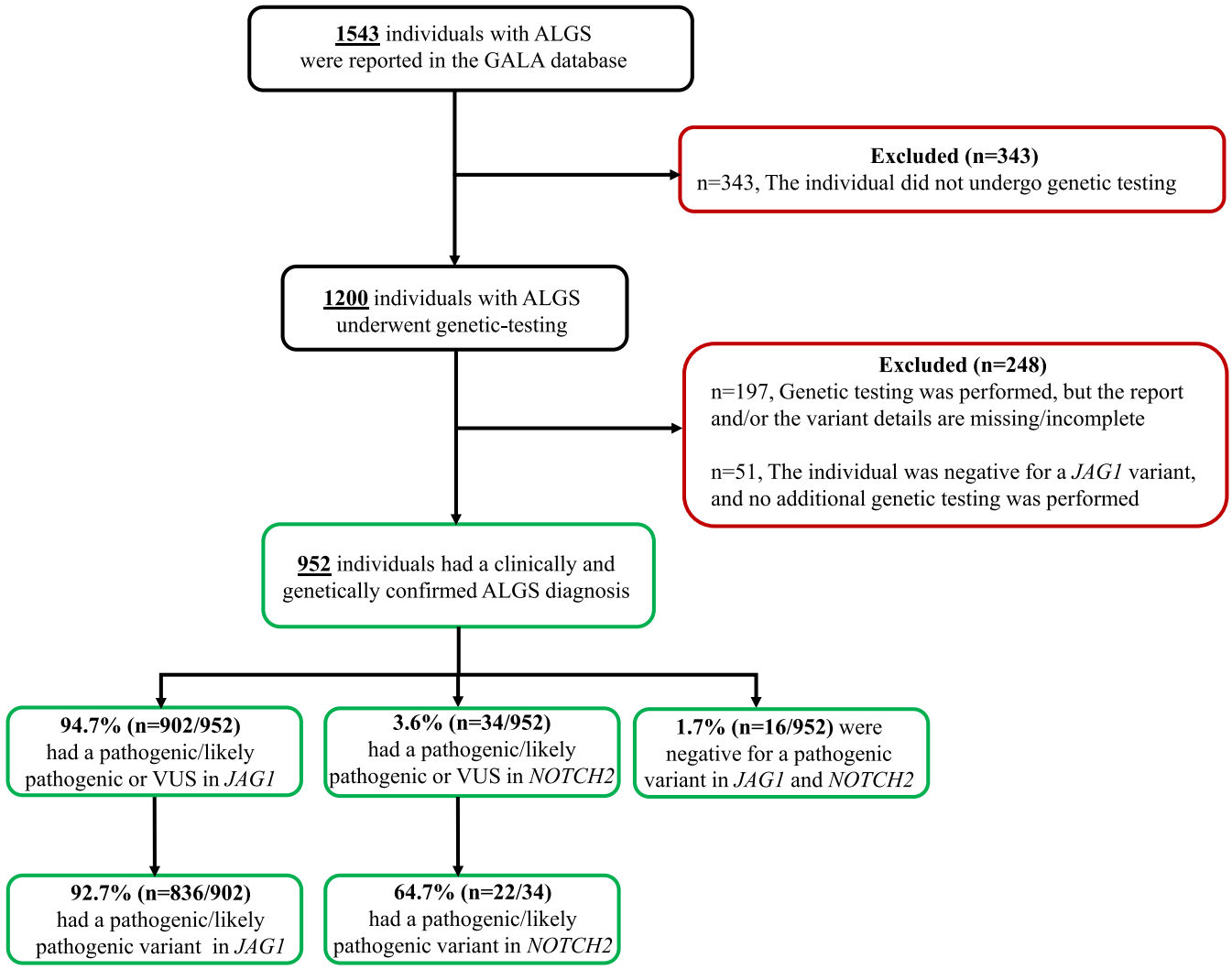

FIGURE 1 | Ascertainment of the GALA study cohort.

3.2 | NOTCH2 Variants in ALGS

Among the 34 *NOTCH2* probands in GALA, 30 unique variants were identified and 18 of these were novel (Table 2). We classified 18/30 variants (60%) as likely pathogenic/pathogenic and 12/30 (40%) as VOUS. Two recurrent variants were identified in the cohort, c.5858G>A; p.Arg1953His ($n=2/34$ probands; 5.9%) and c.6007C>T; p.Arg2003* ($n=4/34$ probands; 11.8%). Both of these variants have been previously reported [23] and were classified as likely pathogenic and pathogenic, respectively. There were no *NOTCH2* structural variants identified.

A total of 61 previously reported *NOTCH2* variants were identified from the literature, the majority of which were missense ($n=41$, 67.2%). Forty variants had been reported as pathogenic/likely pathogenic (65.6%), and 21 (34.4%) were reported as uncertain. We re-assessed all reported variants using recommended ACMG guidelines and disease-specific modifications including the utilisation of gene-specific MAF cut-offs (Table S3) [28, 29]. Reclassification resulted in a drop from likely pathogenic/pathogenic to VOUS for 19 variants (31.1%) and to likely benign for 10 variants (16.4%). One variant (p.Arg1953His) was elevated from VOUS to likely pathogenic (1.6%) and less than half of *NOTCH2* variants (34.4%; $n=21$) retained their original classification. After removing the 10 variants reclassified as likely benign, 51 disease-associated variants remained, of which 20 (39.2%) were classified as pathogenic or likely pathogenic and 31 (60.8%) were classified as VOUS.

With the addition of 18 novel *NOTCH2* variants from GALA, alongside those previously reported as disease-associated from the literature, 69 *NOTCH2* variants are now described in individuals with ALGS (Figure 2A, Table S3). When all 69 variants are considered, the majority of disease-associated *NOTCH2* variants are missense (56.5%, $n=39$), followed by frameshift (15.9%, $n=11$), splice (14.5%, $n=10$) and nonsense (10.4%, $n=7$) (Figure 2A). One synonymous variant (reported here) and one multi-exon deletion have been reported [30].

Notably, the majority of missense variants (79.5%; $n=31/39$) were classified as VOUS. We also observed a bimodal distribution of the missense variants across two hubs, the epidermal growth factor like (EGF-like) domains (61.5%, $n=24$) and the Ankyrin (ANK) repeats (20.5%, $n=8$), with the strongest cluster of EGF-like domain variants localised to the JAG1-binding region (EGF-like 8–12; $n=9$ variants) (Figure 2B).

TABLE 1 | Baseline clinical features for 952 individuals with ALGS.

	All	*JAG1*	*NOTCH2*	Negative for *JAG1* and *NOTCH2*[I]	*p*
n	952	94.7% (*n* = 902)	3.6% (*n* = 34)	1.7% (*n* = 16)	
Male, % (*n*)	56.4% (*n* = 537)	55.7% (*n* = 502)	79.4% (*n* = 27)	50.0% (*n* = 8)	**0.006***
Age at first clinical suspicion (0–1 years), % (*n*)	79.8% (*n* = 751/941)	80.2% (*n* = 715/891)	76.5% (*n* = 26)	62.5% (*n* = 10)	0.803
De novo, % (*n*)	58.2% (*n* = 330/567)	58.9% (*n* = 330/543)	45.0% (*n* = 9/20)	100% (*n* = 1/1)	0.214
Probands, % (*n*)	95.8% (*n* = 912)	95.6% (*n* = 862)	100% (*n* = 34)	100% (*n* = 16)	0.437
Diagnostic criteria, % (*n*)					
Liver involvement, any	98.8% (*n* = 926/937)	98.8% (*n* = 876/887)	100% (*n* = 34)	100% (*n* = 16)	0.660
History of neonatal cholestasis	83.5% (*n* = 768/921)	83.1% (*n* = 727/875)	90.6% (*n* = 29/32)	85.7% (*n* = 12/14)	0.261
Bile duct paucity on first biopsy	64.7% (*n* = 260/402)	64.9% (*n* = 242/373)	56.5% (*n* = 13/23)	83.3% (*n* = 5/6)	0.417
Characteristic facies	89.1% (*n* = 800/898)	90.1% (*n* = 766/850)	57.6% (*n* = 19/33)	100% (*n* = 15/15)	**< 0.001***
Echo-confirmed cardiac anomaly, any	91.1% (*n* = 819/899)	92.2% (*n* = 789/856)	64.3% (*n* = 18/28)	80.0% (*n* = 12/15)	**< 0.001***
Posterior embryotoxon	51.9% (*n* = 413/796)	52.8% (*n* = 399/756)	18.5% (*n* = 5/27)	69.2% (*n* = 9/13)	**< 0.001***
Butterfly vertebrae	43.0% (*n* = 366/852)	44.5% (*n* = 359/806)	3.3% (*n* = 1/30)	37.5% (*n* = 6/16)	**< 0.001***
Renal anomaly, any	38.9% (*n* = 326/837)	39.2% (*n* = 311/794)	34.5% (*n* = 10/29)	35.7% (*n* = 5/14)	0.611
Vascular anomaly, any	37.0% (*n* = 128/346)	37.7% (*n* = 124/329)	21.4% (*n* = 3/13)	25.0% (*n* = 1/4)	0.222

Note: Comparisons were made between those harbouring a *JAG1* or *NOTCH2* variant (P/LP/VOUS). *This denotes statistical significance.

3.3 | NOTCH2 Genotype–Phenotype Analysis in GALA

To study whether *NOTCH2* variant type correlates with disease presentation, all 34 ALGS patients identified in the GALA cohort with a pathogenic/likely pathogenic or VOUS in *NOTCH2* were stratified into three variant groups: protein-truncating (35.2%, *n* = 12), splice site (20.5%, *n* = 7) and non-protein-truncating (44.1%, *n* = 15) and compared. No correlations between *NOTCH2* variant type and ALGS phenotype including presentation of neonatal cholestasis, intrahepatic bile duct paucity and extrahepatic features were identified (Table S4). Available laboratory data from the first year of life, along with the frequency of cholestasis-related complications (such as pruritus and xanthomas), are detailed in Table S5.

We did not identify any differences in NLS or OS at 10–18 years in participants with a history of neonatal cholestasis for all three variant groups (data not shown). To eliminate any confounding effects from including individuals with VOUS, we repeated the analysis including only individuals with a pathogenic/likely pathogenic variant in *NOTCH2* (*n* = 22). In these analyses, our results remained consistent with the primary analysis reported above (data not shown).

Given the high rate of *NOTCH2* variants within the ANK and EGF-like domains, a secondary analysis was performed to determine whether variants clustered in one of these hotspots are associated with a distinct clinical phenotype or prognosis. *NOTCH2*-related ALGS patients with a variant in the ANK or EGF-like domains were clinically and histologically indistinguishable from other patients with *NOTCH2*-related ALGS. There were also no differences between the groups in terms of rates of NLS and OS (data not shown).

3.4 | JAG1 Genotype–Phenotype Analysis in GALA

We report 521 unique *JAG1* variants identified in 863 probands including 244 novel, previously unreported variants (Table S6). The majority of *JAG1* variants are protein-truncating (nonsense, frameshift; 66%, *n* = 342), followed by missense (15.7%, *n* = 80), splicing (13.8%, *n* = 74) and copy number or structural variants (*n* = 24, 4.5%). One previously reported in-frame deletion was also present in our cohort [31]. The incidence of these different mutation types has been reported and has remained relatively unchanged over the past three decades [7, 8, 20, 31]. Moreover, the majority of missense variants identified in our cohort were found within the first six exons (66.3%, *n* = 53/80), a finding that

TABLE 2 | Classification and phenotype analysis of *NOTCH2* variants reported in the GALA study.

Exon/ intron	DNA variant	Protein change	Coding effect	Protein domain	Frequency in gnomAD	Clinical phenotype	Reference	ACMG evidence	Recommended classification
1	c.66dup	p.Ala23Argfs*11	Frameshift	None	Not present	L, F	Novel	PVS1_strong, PM2	Likely pathogenic
Intron 1	c.74-2A>G	p.?	Splice	None	Not present	L	Novel	PVS1_strong, PM2	Likely pathogenic
5	c.857G>C	p.Cys286Ser	Missense	EGF-like 7	Not present	L, F, H, R	Pacheco et al. (2018)	PM1, PM2, PP3	VOUS
6	c.1021dup	p.Asp341Glyfs*37	Frameshift	EGF-like 9	Not present	L	Novel	PVS1_strong, PM1, PM2	Likely pathogenic
6	c.1041del	p.Cys347*	Frameshift	EGF-like 9	Not present	L	Novel	PVS1_strong, PM1, PM2	Likely pathogenic
7	c.1180C>T	p.Pro394Ser	Missense	EGF-like 10	Not present	L, H, V	Kamath et al. (2012)	PM1, PM2, PP3	VOUS
Intron 7	c.1264+1G>C	p.?	Splice	None	Not present	L, F, H	Li et al. (2022)	PVS1_strong, PS3, PM1, PM2	Pathogenic
Intron 7	c.1264+5G>A	p.?	Splice	None	Not present	L, F, H	Novel	PS2_moderate, PM2	VOUS
8	c.1276C>T	p.Pro426Ser	Missense	EGF-like 11	Not present	FDR, L, F	Novel	PM1, PM2, PP3	VOUS
8	c.1418A>T	p.Asp473Val	Missense	EGF-like 12	Not present	L, H, R	Gilbert et al. (2019)	PM1, PM2, PP3	VOUS
8	c.1438T>C	p.Cys480Arg	Missense	EGF-like 12	Not present	FDR, L	Kamath et al. (2012)	PM1, PM2, PP3	VOUS
Intron 9	c.1567+2T>G	p.?	Splice	None	Not present	L, PE, H, R	Novel	PVS1_strong, PM1, PM2	Likely pathogenic
11	c.1750T>A	p.Cys584Ser	Missense	EGF-like 15	Not present	L, H, R	Novel	PS2_moderate, PM1, PM2, PP3	Likely pathogenic
Intron 11	c.1915+2dup	p.?	Splice	None	Not present	L, F, H	Novel	PM2	VOUS
Intron 12	c.2027-1G>A	p.?	Splice	None	Not present	L	Wang et al. (2020)	PVS1_strong, PM1, PM2	Likely pathogenic
16	c.2546_2547del	p.Lys849Argfs*6	Frameshift	EGF-like 22	6.57E-06	L, F, R	Novel	PVS1_strong, PS2_moderate, PM1	Likely pathogenic
16	c.2566_2567del	p.Ser856Leufs*17	Frameshift	EGF-like 22	Not present	L, H	Kamath et al. (2012)	PVS1_strong, PM1, PM2	Likely pathogenic

(Continues)

TABLE 2 | (Continued)

Exon/ intron	DNA variant	Protein change	Coding effect	Protein domain	Frequency in gnomAD	Clinical phenotype	Reference	ACMG evidence	Recommended classification
17	c.2701C>A	p.Pro901Thr	Missense	EGF-like 23	Not present	L, F, PE, BV, H, R	Novel	PM1, PM2, BP4	VOUS
18	c.2765del	p.Asn922Metfs*9	Frameshift	EGF-like 24	Not present	L	Novel	PVS1_strong, PM1, PM2	Likely pathogenic
23	c.3754T>A	p.Cys1252Ser	Missense	EGF-like 32	Not present	FDR, L, H	Novel	PM1, PM2, PP3, BS4	VOUS
31	c.5758G>C	p.Ala1920Pro	Missense	ANK3	Not present	L, F, PE	Togawa et al. (2016)	PM1, PM2, PP3	VOUS
32	c.5858G>A	p.Arg1953His	Missense	ANK4	Not present	L, H	Kamath et al. 2012	PM1, PM2, PM5_ supporting, PP3	Likely pathogenic
32	c.5920G>T	p.Asp1974Tyr	Missense	None	Not present	FDR, L, F	Novel	PM1, PM2, PP3	VOUS
Intron 32	c.5930-2A>G	p.?	Splice	None	Not present	L, F, H, R	Xu et al. (2022)	PVS1_strong, PS3, PS2_ moderate, PM2	Pathogenic
33	c.5983_5984del	p.Leu1995Valfs*29	Frameshift	ANK5	Not present	L, PE, H, R	Liu et al. (2018)	PVS1_strong, PM1, PM2	Likely pathogenic
33	c.6007C>T	p.Arg2003*	Nonsense	ANK5	6.57E-06	L, F, H	Kamath et al. (2012)	PVS1_strong, PS3, PS4, PM1	Pathogenic
33	c.6026A>G	p.Lys2009Arg	Missense	ANK6	Not present	L, F, H	Novel	PS2_moderate, PM1, PM2	Likely pathogenic
33	c.6027G>A	p.Lys2009	Synonymous	ANK6	Not present	L, F, H	Novel	PM1, PM2, BP4	VOUS
34	c.6042_6043del	p.Phe2015Serfs*9	Frameshift	ANK6	Not present	L, F, R	Novel	PVS1_strong, PM1, PM2	Likely pathogenic
34	c.6056G>C	p.Arg2019Pro	Missense	ANK6	Not present	L, F, H	Novel	PS2_moderate, PM1, PM2, PP3	Likely pathogenic

Note: Evidence for a strong impact: PVS1_strong, null allele in a gene where loss of function is the suggested disease mechanism; PS3, well-established in vitro or in vivo functional study supports a damaging effect on the gene or gene product; PS2_moderate, de novo; PM1, located in a mutational hot spot and/or critical or well-established functional domain; PM2, absent form controls (gnomAD); PM5_supporting, novel missense change at an amino acid where a different missense change determined to be pathogenic has been seen before; PP1, co-segregation with disease in multiple affected family members, PP3, multiple lines of computational evidence support a deleterious effect on the gene or gene product. Evidence for a benign impact: BS4, lack of segregation in affected family members.

Abbreviations: ANK, ankyrin; BV, butterfly vertebra; EGF, epidermal growth factor; F, facies; FDR, first-degree relative with a history of ALGS; H, heart; L, liver; LNR, Lin12-Notch repeat; PE, posterior embryotoxon; R, renal; V, vascular.

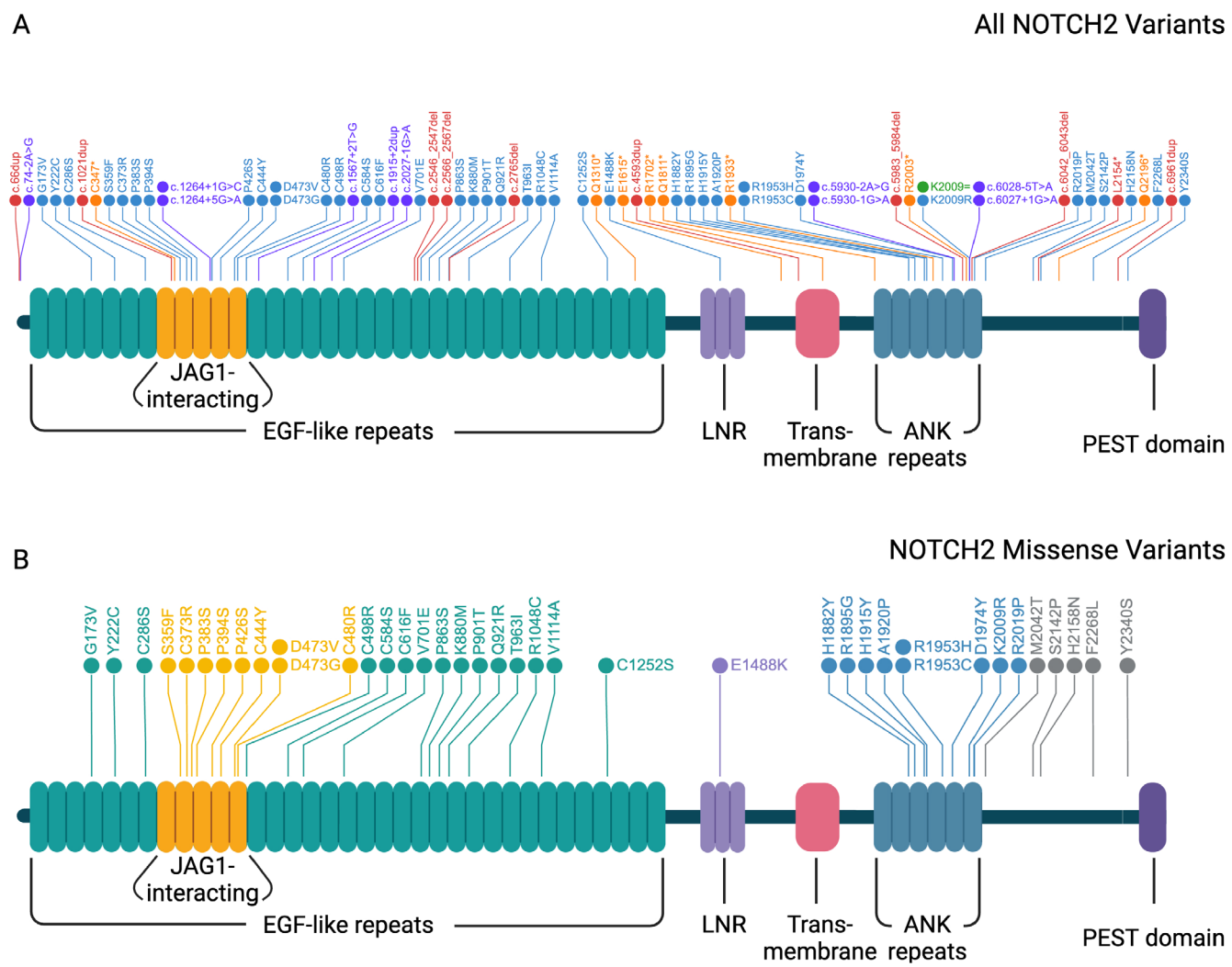

FIGURE 2 | *NOTCH2* variants reported for ALGS. (A) All disease-associated *NOTCH2* variants (VOUS, likely pathogenic, and pathogenic; $n = 69$) identified in this study and those previously reported are plotted along the *NOTCH2* protein. Structural variants and those predicted to have a benign effect on protein function are not included ($n = 1$). Variants are colour-coded to distinguish variant types: red (frameshift, $n = 11$), blue (missense, $n = 33$), orange (nonsense, $n = 7$), purple (splice, $n = 10$), and green (silent, $n = 1$). Protein domains are depicted using the following colour scheme: teal (EGF-like), yellow (JAG1-interacting, EGF-like), light purple (Lin-12/Notch repeat; LNR), red (transmembrane), blue (ankyrin repeats) and dark purple (PEST domain). (B) All disease-associated missense *NOTCH2* variants identified in this study and those previously reported (VOUS, likely pathogenic, and pathogenic; $n = 33$) are plotted along the *NOTCH2* protein. Variants are colour-coded according to their location in a functional domain: teal (EGF-like), yellow (JAG1-interacting EGF-like), blue (ankyrin repeats) and grey (no domain). The image was created using Protein Paint (https://proteinpaint.stjude.org/) and BioRender.

has also been previously reported [4, 7, 32]. Protein-truncating and full or partial gene deletions are anticipated to result in loss of function (LoF) and were all classified as likely pathogenic or pathogenic when disease pathogenesis (haploinsufficiency), inheritance and absence in unaffected individuals (i.e., gnomAD) were taken into account [28, 33]. Using disease-specific modified ACMG classification criteria (Table S2), we classified 56 (70%) unique *JAG1* missense variants as pathogenic or likely pathogenic and 24 (30%) as VOUS (Table S6).

Given the size of our cohort, we were able to investigate the frequency of recurrent variants. The most common recurrent variant type in *JAG1*-ALGS are whole gene deletions, which occur in 6.4% of probands ($n = 55/863$ *JAG1* probands). Whole gene deletions occur with varying breakpoints with no evidence of regions with increased vulnerability to breakage, as previously reported [34]. Within 784 probands harbouring a single nucleotide or insertion–deletion (indel) variant, we report 496 unique variants. The majority of these variants were seen in only a single proband (82.5%, $n = 409$), whereas 17.5% of variants ($n = 87$) were identified in two or more probands. Most of these recurrent variants are found in repetitive or homopolymeric regions and/or in less than five probands. The most commonly occurring variant was a frameshift, c.2122_2125del (p.Gln708Valfs*34) (3.6% of probands with a single nucleotide or indel variant, $n = 28$), which involves the deletion of CAGT within a tandem repeat (CAGTCAGT). Overall, 16 variants were seen at a frequency greater than 1%.

To study whether variant type correlates with a specific ALGS phenotype, all participants with a pathogenic, likely pathogenic, or VOUS in *JAG1* were stratified into four groups: protein-truncating variant ($n = 538$), splice site variants ($n = 151$), missense ($n = 133$), or structural variant ($n = 82$). No association was identified between variant type and clinical phenotype including the presentation of neonatal cholestasis, intrahepatic bile duct paucity, or extrahepatic features (Table S5). We again did not identify any differences in NLS or OS at 10- and 18-year-olds in participants with a history of neonatal cholestasis for all four variant groups (Figure S1A,B). To avoid overinterpretation of these findings, participants with a VOUS were removed, and the analysis was repeated with results remaining unchanged (data not shown).

3.5 | Characterisation of Phenotypic Differences Between Individuals With JAG1 and NOTCH2 Variants in GALA

We investigated phenotypic differences between individuals harbouring *JAG1* ($n = 902$) or *NOTCH2* ($n = 34$) variants in the GALA cohort (Table 1). The two groups were comparable in terms of liver involvement, renal anomalies and vascular involvement. However, *NOTCH2*-associated ALGS participants were significantly less likely to have characteristic facies ($p < 0.001$), an ECHO-confirmed cardiac anomaly ($p < 0.001$), posterior embryotoxon ($p < 0.001$) and butterfly vertebrae ($p < 0.001$), compared to participants with *JAG1*-associated ALGS. Moreover, *NOTCH2*-associated ALGS participants were significantly more likely to be male compared to *JAG1*-associated ALGS participants ($p < 0.006$). A comparison of NLS rates at 10 and 18 years among individuals with ALGS presenting with neonatal cholestasis found no statistically distinguishable difference between those with a *NOTCH2* or *JAG1* variant (log-rank $p = 0.0192$; Figure 3A). In line with the analysis of NLS, OS rates at 10 and 18 years were comparable (log-rank $p = 0.506$; Figure 3B). To confirm the robustness of these findings, participants with a VOUS in either gene were removed, and the analysis was repeated, yielding the same results (data not shown).

3.6 | Molecularly Uncharacterized ALGS Individuals in GALA

No pathogenic variants were detected in either *JAG1* or *NOTCH2* for 1.7% ($n = 16/952$) of the patients within our cohort, despite their meeting clinical criteria for ALGS. Hepatic involvement was universally reported in these patients, and the frequency of extrahepatic manifestations was comparable to ALGS patients with *JAG1* variants (Table 1). Notably, bile duct paucity was reported in 83.3% ($n = 5/6$) of mutation-negative probands. NLS of molecularly characterised (presence of a *JAG1* or *NOTCH2* variant; $n = 753$) and uncharacterised (absence of a *JAG1* or *NOTCH2* variant; $n = 12$) individuals with ALGS who presented with neonatal cholestasis were comparable at 10- and 18-years (log-rank, $p = 0.411$; Figure S2A). Similarly, an analysis of OS including all individuals, both molecularly characterised ($n = 931$) and uncharacterised ($n = 16$), at 10 and 18 years yielded comparable results (log-rank, $p = 0.139$; Figure S2B).

4 | Discussion

We present data from an international cohort of 952 individuals with clinically confirmed ALGS diagnoses from 66 participating institutes across 29 countries, all of whom have undergone genetic testing. We report a slightly higher incidence of *NOTCH2* variants (3.6%) in ALGS than has been previously reported (2.5%), which is likely due to the greater size and geographical distribution of our cohort [7]. We identified and classified 18 novel *NOTCH2* variants, which increases the number of reported variants to date by 30%, and we present 244 novel *JAG1* variants ($n = 222$ pathogenic/likely pathogenic, $n = 22$ VOUS). Our findings demonstrate a statistically supported phenotypic divergence between patients with *JAG1* and *NOTCH2* variants. These phenotypic differences should be considered during clinical evaluation for ALGS, particularly for patients with isolated cholestasis who have not yet undergone molecular testing.

Our reference catalogue of all reported *NOTCH2* variants associated with ALGS obtained through meticulous curation and rigorous disease-specific variant interpretation will aid in the evaluation of variants when they are identified in the clinic. For our classification framework, we reviewed all evidence guidelines provided by the ACMG and modified them when applicable based on our expertise with ALGS genetics, which included the adaptation of gene-specific MAF cut-offs for *JAG1* and *NOTCH2* based on disease incidence and gene-specific disease frequency [28, 29]. For *NOTCH2*, this cut-off corresponds to an absence of alleles in gnomAD (v.2.2.1. This adaptation is supported by a recent study that stratified 35 patients with *NOTCH2* variants by variant frequency and found a statistically significant correlation between variants that were absent in gnomAD and those that were both predicted to be damaging by *in silico* models and were present in patients with high GGT levels [35]. Review of all *NOTCH2* variants in the GALA cohort ($n = 30$) resulted in 18 likely pathogenic/pathogenic and 12 VOUS (40%) classifications. *NOTCH2* VOUS rates vary drastically by reporting centre and phenotypic diversity of the tested population. Our VOUS rate of 40% is lower than what is reported in ClinVar (62.9%), which is likely attributable to the highly phenotyped nature of our GALA cohort [1]. Our study inclusion criteria for individuals with a VOUS required the presence of three ALGS disease features rather than the two required for inclusion of individuals with a likely pathogenic or pathogenic variant. This increased stringency for inclusion of VOUS was done with purpose to retain only those variants with a high likelihood of disease relevance. For this reason, all VOUS were included in statistical analyses within the GALA cohort. Our analysis of previously reported *NOTCH2* variants in the literature indicates that more than half (65.6%, $n = 40$ out of 61) were misclassified, of which the majority (85%, $n = 34$ out of 40) resulted in a drop in classification from likely pathogenic/pathogenic to VOUS or likely benign or VOUS to likely benign.

Missense variants were the predominant variant type for *NOTCH2*, and were largely classified as VOUS within the GALA study (69.2%, nine out of 13). Our findings, alongside previously reported missense variants in *NOTCH2*, strongly support the presence of two mutational hotspots, one occurring within the EGF-like domains and a second occurring within the ANK

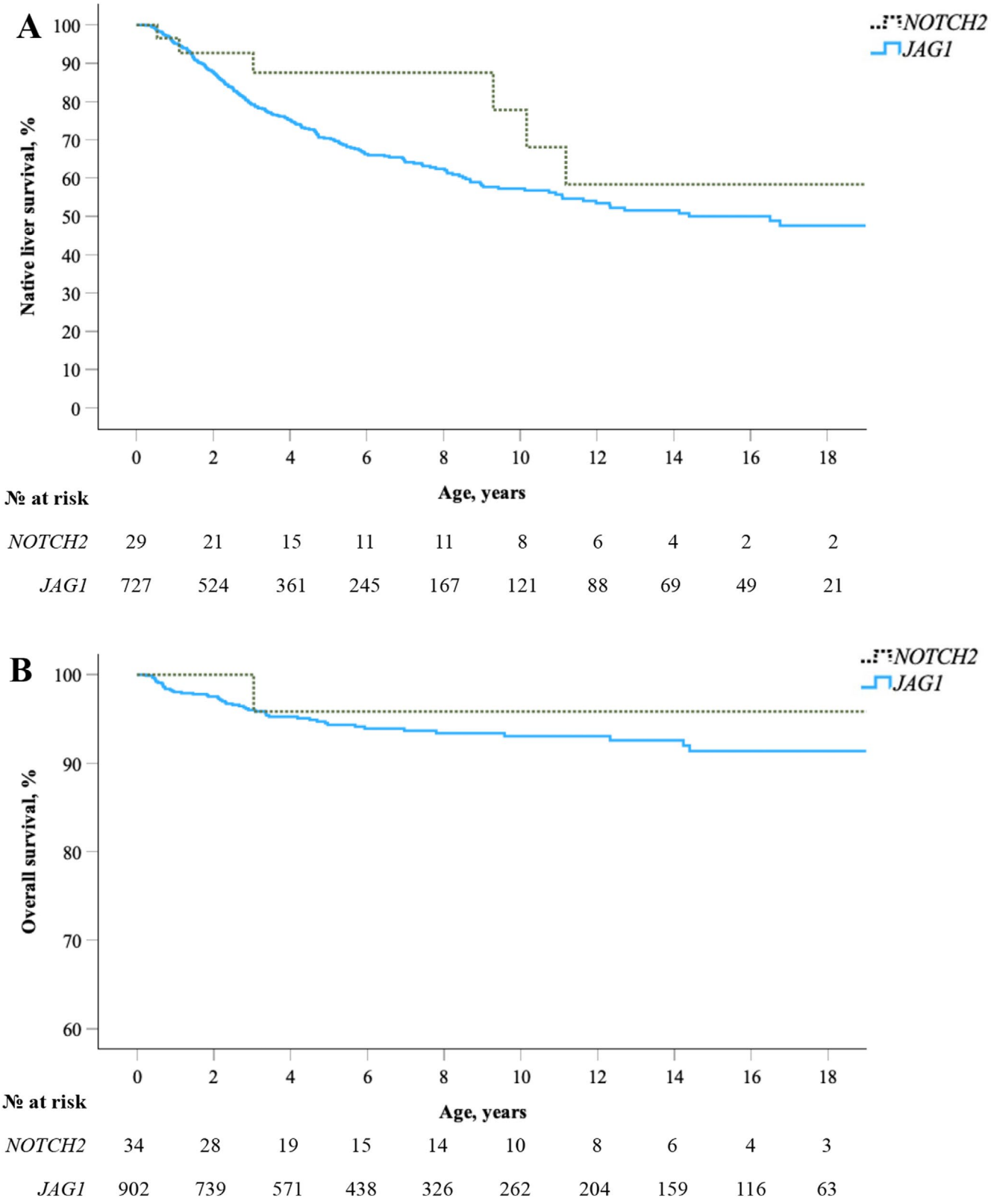

FIGURE 3 | Native liver survival (NLS) and overall survival (OS) rates in individuals with ALGS with a *JAG1* or *NOTCH2* variant. (A) Comparable rates of 10 and 18-year native liver survival were observed among ALGS patients with a *NOTCH2* ($n = 29$) or *JAG1* ($n = 727$) variant who presented with neonatal cholestasis (log-rank $p = 0.192$). (B) 10 and 18-year survival rates for both *NOTCH2* ($n = 34$) and *JAG1*-ALGS ($n = 902$) individuals were found to be statistically indistinguishable (log-rank $p = 0.506$).

repeats. Nearly all of the identified *NOTCH2* missense variants (GALA study combined with previously reported) are reported in these two functional regions (34/39; 87.2%), with over half (76.5%; $n = 26$ out of 34) found within the EGF-like domains. Identification of these two mutational hot spots can aid in predicting the pathogenicity of variants in *NOTCH2*.

The determination of whether a DNA change occurring within *JAG1* or *NOTCH2* is causal for ALGS depends on multiple factors. The ACMG has published recommendations to help in the interpretation of disease causality for variants [28], but these generalised guidelines often require disease-specific modifications based on deep knowledge of mutation type and disease mechanism. The published guidelines provide criteria that are assessed individually for each variant and used to establish a classification of pathogenic, likely pathogenic, VOUS, likely benign, or benign. Each criterion is assigned a pre-determined weight of very strong, strong, moderate, or supporting, and this evidence is tallied to arrive at a final classification for each variant. Our analysis of the pathogenicity of *JAG1* and *NOTCH2* variants relied predominantly on four classification criteria outlined by the ACMG [28], which we found to be most informative for or against disease causality (detailed in Table S2). We found that low frequency or absence of a variant in control populations was applicable to nearly all analysed variants (98.9% of probands) and provided moderate support for pathogenicity. Evidence supporting a damaging effect on protein function was found to be highly important for variant classification. A recent study reporting on the functional effects of nearly 3000 *JAG1* variants within exons 1–7 provided evidence in support of pathogenicity for 23 *JAG1* variants reported here [36]. Only six *NOTCH2* variants have been studied at the protein level, all of which showed abnormal function and are classified here as likely pathogenic or pathogenic [23]. Additional functional studies for both *JAG1* and *NOTCH2* will be important in resolving the pathogenicity of VOUS. We also recommended a reduction in the suggested weight for two additional ACMG criteria. We recommend that the identification of a *de novo* variant be considered as moderate, rather than strong, evidence toward pathogenicity since inheritance status is not critical in ALGS, where variable expressivity is highly prevalent, and where the same variant can be *de novo* in one family and inherited in a different family. We also recommend that the identification of a novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before should be weighted as supporting rather than as moderate evidence. In the functional study described above by Gilbert et al. [36], the authors found no correlation between abnormal function of one missense change extending to all other substitutions at that amino acid residue. We expect that as more research emerges for *JAG1* and *NOTCH2* these guidelines can be further modified and improved to help support clear and appropriate variant classifications for ALGS.

Given the size of our cohort, we were able to define significant phenotypic differences between *JAG1*- and *NOTCH2*-related ALGS. *NOTCH2*-related ALGS patients were significantly less likely to have butterfly vertebrae and characteristic facies, findings that are consistent with an earlier, small case series [23]. These data suggest that NOTCH2 may not be expressed in developing vertebral bodies and or may have a distinct role in regulating craniofacial bone development. In contrast, loss of *JAG1* function in mesenchymal progenitors leads to opposing effects in cortical and trabecular osteoblasts, which has been suggested to contribute to the skeletal phenotype in *JAG1*-related ALGS patients [37, 38]. Moreover, studies in both zebrafish and mice support this finding, with loss of *JAG1* expression resulting in greater malformations of the inner and middle ear bones than loss of *NOTCH2* [39]. Our study further extends the observations of Kamath et al. [23] and reports significantly reduced penetrance of other extrahepatic manifestations in *NOTCH2*-related ALGS patients including cardiac and eye anomalies. We also identified a marked male predominance among *NOTCH2*-related ALGS patients. Male predominance in aortic valve disease, an unrelated condition that is caused by LoF variants in *NOTCH1*, has also been reported, raising the possibility of a shared mechanism between these two Notch signalling disorders [32, 40]. We postulate that Notch signalling could regulate sex steroid hormones and this interaction could account for the observed sex difference. Liver involvement was observed in all *NOTCH2*-related ALGS patients, however our cohort was biased as all individuals were ascertained from liver clinics. Regardless, the presentation of liver disease and rates of NLS and OS were comparable among all participants. Taken together, these data suggest that reliance on classical clinical phenotypic definitions of ALGS may miss patients with *NOTCH2*-related disease, or lead to misdiagnosis with biliary atresia, and that expansion of genetic testing criteria may improve diagnostic accuracy, particularly in cases with atypical ALGS phenotypes. Standard genomic diagnostic workflows typically involve simultaneous sequencing and deletion/duplication analysis of both *JAG1* and *NOTCH2*, often due to their presence on cholestatic panels, which also include many other genes associated with cholestasis [41]. The use of these panels is standard of care in most liver clinics. Moreover, despite these findings, the true spectrum of the clinical phenotype associated with *NOTCH2* variants remains to be fully elucidated as this study focuses on those with an ALGS-like phenotype, and further investigation will be necessary to determine if non-characteristic ALGS features can be present in these individuals. It is important to note that *NOTCH2*-related ALGS is extremely rare, which results in small cohort sizes that could impact the robustness of findings. We will validate our observations in the future as our cohort size increases with additional patient enrollment.

No genotype–phenotype differences in variant type were identified for individuals with *JAG1* or *NOTCH2* variants, which is in agreement with previous studies [19–21]. Although testing strategies may differ across participating centres, it is unlikely that this would influence the detection and classification of variants. *JAG1* and *NOTCH2* are the only two genes implicated in ALGS, and genome sequencing studies on ALGS individuals in whom a *JAG1* or *NOTCH2* mutation has not been identified have failed to implicate novel genes [30]. More likely, the variable expressivity of ALGS is due to the contribution of genetic modifiers, epigenetic mechanisms, or environmental factors in disease severity. Four candidate genetic modifiers have been implicated in the pathogenesis or amelioration of *JAG1*-related liver disease, both in mouse models and humans [15–18], and these types of genetic modifier studies have not yet been extended to *NOTCH2*-related disease. It is possible that non-genetic factors could play a role in modulating disease penetrance. Two studies in monozygotic

twins have proposed the role of prenatal hypoxia as an influencing factor in ALGS disease severity due to unequal blood flow and twin-to-twin transfusion syndrome [12, 14]. The contribution of non-genetic modifiers to ALGS disease severity has not been studied to the same degree as genetic modifiers. Collectively, these results, along with previous publications, illustrate a complex underlying molecular aetiology of ALGS and support further investigations into modifiers of Notch signalling.

A pathogenic variant was not identified in *JAG1* and *NOTCH2* for 1.7% of individuals in the study. A prior study in a large ALGS cohort reported a pathogenic variant negative rate of 3.2% [7]. When genome sequencing was performed in this cohort of patients, four novel pathogenic variants were identified in *JAG1* ($n=3$) and *NOTCH2* ($n=1$), indicating that the application of additional sequencing technologies was able to increase the diagnostic yield [30]. As sequencing technologies and bioinformatic methodologies advance in conjunction with our understanding of how non-coding regions influence *JAG1* and *NOTCH2* expression, we imagine that we might be able to identify novel variations in patients with molecularly uncharacterised ALGS. Alternatively, a thorough investigation of the clinical phenotypes in these patients could help point to other molecular diagnoses, and careful tracking of evolving clinical features will be critical in these individuals.

5 | Conclusion

Our reference catalogue summarises 79 *NOTCH2* variants, of which 69 are associated with ALGS. This catalogue serves as an invaluable resource for clinicians and clinical laboratory geneticists, facilitating interpretation and classification of *NOTCH2* variants. Our comprehensive literature review revealed that the majority of reported disease-causing *NOTCH2* variants were later reclassified as either VOUS or likely benign. This observation underscores the importance of employing strict clinical genotyping and utilising disease-specific variant classification criteria when assessing variants. In the GALA cohort, we identified 18 novel *NOTCH2* variants and corroborated earlier findings of a predominance of missense variants in two hubs along the *NOTCH2* gene. Furthermore, we clearly establish phenotypic differences between patients with *NOTCH2* variants compared to those with *JAG1* variants. These data suggest that reliance on classical clinical phenotypic definitions of ALGS may miss patients with *NOTCH2*-related disease and that an inclusive approach to genetic testing is critical for diagnosis.

Author Contributions

All authors contributed to data collection, analysis and interpretation, writing and review of the manuscript. Shannon M. Vandriel, Kathleen M. Loomes, Nancy B. Spinner, Melissa A. Gilbert and Binita M. Kamath contributed to the design of the study, had access to and verified the data, and were responsible for the decision to submit the manuscript.

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Acknowledgements

The authors would also like to acknowledge Desiree Vaz and Mila Valcic from the GALA DCC at the Hospital for Sick Children. The authors would also like to thank all local research teams who helped with data collection.

Conflicts of Interest

Shannon M. Vandriel [consults for Mirum Pharmaceuticals Inc.], Li-Ting Li, Huiyu She, David A. Piccoli, Irena Jankowska, Piotr Czubkowski, Dorota Gliwicz-Miedzińska, Emanuele Nicastro, Dominique Debray, Étienne M. Sokal, Tanguy Demaret, Rima L. Fawaz, Silvia Nastasio, Kyung Mo Kim, Seak Hee Oh, Catherine Larson-Nath [nothing to disclose], Sahana Shankar, Shikha S. Sundaram, Alexander Chaidez, Pinar Bulut, Pier Luigi Calvo, Mureo Kasahara, Niviann Blondet, Eberhard Lurz, Anna-Maria Kavallar, Emmanuel M. Gonzales, Jérôme Bouligand, Jeffrey A. Feinstein, Susan M. Siew, Michael O. Stormon, Rene Romero, M. Kyle Jensen, Catalina Jaramillo, James E. Squires, Sarah M. Bedoyan, Jane Hartley, Way Seah Lee, Chatmanee Lertudomphonwanit, Henry C. Lin, Yael Mozer-Glassberg, Amin J. Roberts, Helen M. Evans, Gabriella Nebbia, Pamela L. Valentino, Jesus Quintero Bernabeu, Cigdem Arikan, María Legarda Tamara, Cristina Molera Busoms, Thomas Damgaard Sandahl, Andréanne N. Zizzo, Aglaia Zellos, Ruben E. Quiros-Tejeira, Ermelinda Santos-Silva, Jernej Brecelj, Maria Camila Sanchez, Maria Lorena Cavalieri, Christos Tzivinikos, Sabina Wiecek, John Eshun, Zerrin Önal, Cristina Gonçalves, Jennifer Garcia, Seema Alam, Carolina Jimenez-Rivera, Luis Bujanda [nothing to disclose], Jian-She Wang [Consultant for Ipsen Biopharmaceuticals Inc., Mirum Pharmaceuticals Inc., and Qing Bile Therapeutics Inc.], Kathleen M. Loomes [Consultant for Mirum Pharmaceuticals Inc., Ipsen Biopharmaceuticals Inc., and Travere Therapeutics Inc.], Lorenzo D'Antiga [Consultant for Mirum Pharmaceuticals Inc., Ipsen Biopharmaceuticals Inc., Alexion, Astra Zeneca, Vivet Therapeutics, Spark, Genespire, Tome, Advanza Pharma], Florence Lacaille [Consultant for Alexion], Björn Fischler [Consultant for Ipsen Biopharmaceuticals Inc.], Henrik Arnell [consultant for Ipsen Biopharmaceuticals Inc., Baxter, Mirum Pharmaceuticals Inc.], Winita Hardikar [Consultant for Ipsen Biopharmaceuticals Inc., Advanz Pharma], Emmanuel Jacquemin [Consultant for CTRS, Theravia and Vivet Therapeutics, France], Noelle H. Ebel [Consultant for Mirum Pharmaceuticals Inc.], Saul J. Karpen [Consultant for Ipsen Biopharmaceuticals Inc., Intercept, Mirum Pharmaceuticals Inc. and Vertex], Deirdre A. Kelly [European Advisory Board for Mirum Pharmaceuticals Inc.; Advisory Board for Intercept & Advanz Pharma], Henkjan J. Verkade [Consultant for Ipsen Biopharmaceuticals Inc., Intercept, Mirum Pharmaceuticals Inc., Orphalan and Vertex], Ryan T. Fischer [Speaker and Consultant for Ipsen Biopharmaceuticals Inc. and Mirum Pharmaceuticals Inc.], Nathalie Rock [Consultant for Mirum Pharmaceuticals Inc.], Wikrom Karnsakul [Consultant for Mirum Pharmaceuticals Inc., Ipsen Biopharmaceuticals Inc., and Travere Therapeutics Inc.], Victorien M. Wolters [Speaker for

Mirum Pharmaceuticals Inc.], Amal A. Aqul [Consultant for Mirum Pharmaceuticals Inc., Ipsen Biopharmaceuticals Inc., and Sarepta], Giuseppe Indolfi [Consultant for Mirum Pharmaceuticals Inc.], Kathleen B. Schwarz [Research grant Ipsen Biopharmaceuticals Inc. and Consultant, Mirum Pharmaceuticals Inc], Nanda Kerkar [Advisory Board for Ipsen Biopharmaceuticals Inc. and Mirum Pharmaceuticals Inc.], Quais Mujawar [Advisory Board for Alexion Pharma], Richard J. Thompson [Consultant for Shire, Ipsen Biopharmaceuticals Inc., Mirum Pharmaceuticals Inc., Horizon Pharmaceuticals, Sana Biotechnology, GenerationBio, Retrophin and Qing Bile Therapeutics Inc.], Bettina E. Hansen [Consultant for Mirum Pharmaceuticals Inc., Ipsen Biopharmaceuticals Inc., Chemomab, Calliditas, Intercept, Cyma Bay, unrestricted grants from Cyma bay, Intercept, Mirum Pharmaceuticals Inc., and Ipsen Biopharmaceuticals Inc.], Nancy B. Spinner [Consultant for Mirum Pharmaceuticals Inc., and Travere Therapeutics Inc.], Melissa A. Gilbert [Consultant for Travere Therapeutics Inc.], Binita M. Kamath [Consultant for Mirum Pharmaceuticals Inc., Ipsen Biopharmaceuticals Inc., Third Rock Ventures and Audentes Therapeutics, unrestricted educational grants from Mirum Pharmaceuticals Inc., and Ipsen Biopharmaceuticals Inc.].

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Eligibility criteria for the GALA study. **Table S2:** Modified American College of Medical Genetics (ACMG) criteria. **Table S3:** Reclassification of all *NOTCH2* variants reported in the literature including those identified within the GALA cohort. **Table S4:** Baseline clinical characteristics of 34 individuals (100% probands) diagnosed with ALGS and carrying a *NOTCH2* variant (P/LP/VOUS), categorised by variant type. **Table S5:** All *JAG1* variants reported in the GALA cohort ($n = 902$). **Table S6:** Baseline clinical characteristics of 902 individuals (95.6% probands) diagnosed with ALGS and harbouring a *JAG1* variant (P/LP/VOUS), stratified by variant type. **Figure S1:** Native liver survival (NLS) and overall survival (OS) rates in individuals with a *JAG1* variant with intergenotype comparisons. **Figure S2:** Comparable rates of native liver survival (NLS) and overall survival (OS) in individuals with ALGS with a molecularly characterized or uncharacterized diagnosis.