# Novel frameshift variant (c.409dupG) in *SLC25A38* is a common cause of congenital sideroblastic anaemia in the Indian subcontinent

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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jclinpath-2020-206647).

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Received 11 April 2020
Revised 3 June 2020
Accepted 5 June 2020
Published Online First 30 June 2020



**To cite:** Ravindra N, Athiyarath R, S E, *et al*. *J Clin Pathol* 2021;**74**:157–162.

## ABSTRACT

**Aims** Congenital sideroblastic anaemias (CSAs) are a group of rare disorders with the presence of ring sideroblasts in the bone marrow. Pathogenic variants are inherited in an autosomal recessive/X-linked fashion. The study was aimed at characterising the spectrum of mutations in *SLC25A38* and *ALAS2* genes in sideroblastic anaemia patients, exploring the genotype-phenotype correlation and identifying the haplotype associated with any recurrent mutation.

**Patients and methods** Twenty probable CSA patients were retrospectively analysed for genetic variants in *ALAS2* and *SLC25A38* genes by direct bidirectional sequencing. Real-time PCR was used to quantify gene expression in a case with promoter region variant in *ALAS2*. Three single nucleotide polymorphisms were used to establish the haplotype associated with a recurrent variant in the *SLC25A38* gene.

**Results** Six patients had causative variants in *ALAS2* (30%) and 11 had variants in *SLC25A38* (55%). The *ALAS2* mutated cases presented at a significantly later age than the *SLC25A38* cases. A frameshift variant in *SLC25A38* (c.409dupG) was identified in six unrelated patients and was a common variant in our population exhibiting 'founder effect'.

**Conclusion** This is the largest series of sideroblastic anaemia cases with molecular characterisation from the Indian subcontinent.

## INTRODUCTION

Sideroblastic anaemias constitute a heterogeneous group of congenital as well as acquired anaemias characterised by the presence of ring sideroblasts (RS) in the bone marrow. The congenital sideroblastic anaemias (CSAs) are rare and caused by variants in numerous genes involved in iron transport and metabolism; such as haem and iron-sulphur [Fe-S] cluster biosynthesis and mitochondrial protein production.[1] Disruption of these pathways results in mitochondrial iron accumulation, which is visualised as siderotic granules in erythroid precursor cells.

The inheritance of CSA is X-linked (XLSA) or autosomal recessive. Affected cases may exhibit isolated features of anaemia (non-syndromic) or may have a constellation of associated anomalies (syndromic). Non-syndromic sideroblastic anaemias are more common; with *ALAS2* (XLSA) being the first gene to be implicated in this category.[2] Variants in *SLC25A38* were identified in cases that were phenotypically similar to XLSA but inherited in an autosomal recessive pattern.[3] Other rare forms of non-syndromic CSA are caused by variants in *GLRX5* and *HSPA9*.[4 5] *GLRX5* is vital for [Fe-S] biosynthesis and maintenance of mitochondrial and cytosolic iron homoeostasis.[6] All published cases diagnosed with variants in *GLRX5* have presented in adulthood.[4 7]

Most patients present with symptomatic anaemia requiring transfusions. Syndromic sideroblastic anaemias with features including neurological symptoms have been described with specific genetic aetiologies related to *ABCB7*, *SLC19A2*, *PUS1*, *YARS2* and *NDUFB11* genes.[8] Recently, a syndrome complex comprising sideroblastic anaemia, primary B-cell immunodeficiency, periodic fevers and developmental delay has been described; which is caused by variants in *TRNT1* that encodes a crucial enzyme required in the maturation of nuclear and mitochondrial transfer RNAs.[9 10]

Ring sideroblasts are not pathognomonic of CSA and are found in some subtypes of myelodysplastic syndromes (MDS)—MDS with ring sideroblasts or MDS/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis.[11] These entities are associated with somatic variants in *SF3B1*.[12] Sideroblastic anaemia can also be drug/toxin-related and can be caused by ethanol, isoniazid, chloramphenicol, lead, zinc or by nutritional deficiencies of pyridoxine or copper.[11 13]

With rapid advances in the field of molecular diagnostics over the last two decades, it is now possible to identify causative genes/variants in more than two-thirds of CSAs.[14] Studies of CSA from the Indian subcontinent are few and limited to a few short case series/reports; often with no genetic analysis.[15 16] Genetic testing in CSA establishes congenital aetiology and simultaneously excludes MDS that can potentially transform into leukaemia.[17] Unlike XLSA which responds to pyridoxine, CSA caused by variants in *SLC25A38* is refractory to pyridoxine treatment. Demonstrating the causative mutation in the proband can also aid in prenatal testing in subsequent pregnancies. In this study, we report cases of CSA from the Indian subcontinent with clinical and molecular characterisation.

## SUBJECTS AND METHODS

### Cases

This is a retrospective study of cases who opted for molecular analysis and presented with the following

features: unexplained anaemia with bone marrow examination showing features of sideroblastic anaemia (presence of ring sideroblasts) after ruling out any secondary causes for sideroblast formation. Peripheral blood was collected from the probands and the parents and/or siblings wherever possible.

### Molecular analysis

DNA was extracted from peripheral blood by Gentra Puregene blood DNA kit (QIAGEN Sciences, Maryland, USA). PCRs for *ALAS2* (NM_000032.5) and *SLC25A38* (NM_017875.4) genes were performed using primers designed and optimised in-house (online supplementary data 1–4). The primers were designed to cover all exons and splice site regions of both genes and part of the promoter sequences of *ALAS2*. Bi-directional sequencing reaction was carried out by using BigDye Terminator v1.1 cycle sequencing kit according to manufacturer instructions (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) and analysed by ABI Prism 3130 Genetic Analyser (Thermo Fisher Scientific). The causative genetic variant was also confirmed in the parents' samples wherever available. Variants were classified and annotated according to Human Genome Variation Society nomenclature.

### In silico analysis

Online bioinformatics tools: MutationTaster (http://www.mutationtaster.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/index.php) and Human Splicing Finder (http://www.umd.be/HSF/) were used to predict the consequences of single nucleotide and splice junction variants. We also compared variants obtained in our study with previously published reports and databases for inherited genetic diseases (ClinVar and HGMD). Pathogenicity was ascribed as per recommendations by the American College of Medical Genetics and Genomics (ACMG) and the Association of Molecular Pathology.[18]

### Haplotype construction

Three single nucleotide polymorphisms (SNPs) in linkage were identified throughout the *SLC25A38* gene: rs2270770 in exon-2, rs1995236 in intron-4 and rs870843 in intron-5 (figure 1). These were used to construct a haplotype associated with the frameshift variant c.409dupG. A total of 16 mutated alleles including five patients who were homozygous, one patient who was compound heterozygous and five parental samples with carrier status for the above-mentioned variant were considered. The disease-associated haplotype was compared with CSA cases exhibiting other mutations and normal controls, for a total of 32 'wild-type' alleles.

### Characterisation of promoter variant in *ALAS2*

Reticulocytes were isolated from the patient and mother who exhibited promoter variant in *ALAS2* (c.-111G>A). Quantitation of the *ALAS2* expression was done by real-time quantitative PCR (RQ-PCR) and confirmed with controls.

### Genotype-phenotype correlation and statistical analysis

The following clinical/laboratory parameters were applied for comparison between the two groups (*ALAS2* mutated vs *SLC25A38* mutated): age at presentation, haemoglobin levels at presentation, reticulocyte count and percentage of ring sideroblasts in bone marrow aspirates. Descriptive statistics and statistical significance calculated by Fisher's exact test and Mann-Whitney U test were performed using IBM SPSS software (V.16.0.0).

## RESULTS

### Clinical and haematological parameters

Twenty cases with suspected inherited sideroblastic anaemia were referred for genetic testing in the study period. History of consanguineous marriage was elicited in 45.5% of families included in this study. The median age at presentation was 19 months (range: 0.25 to 684 months). Male patients constituted the majority of cases in our cohort (16/20; 80%). The male: female ratio was 4:1. The mean haemoglobin at presentation was 51 g/L (SD: 1.96). The median ring sideroblast percentage was 40.5% (range: 20% to 98%) and the median reticulocyte count was 0.91% (range: 0.19% to 4.3%).

### Molecular analysis

Sequencing identified variants in *ALAS2* in six male patients (30%) and in *SLC25A38* in 11 patients (55%). Nine of the *SLC25A38* mutated cases were male. No pathogenic variants in either gene were seen in the remaining three patients. All six variants found in the *ALAS2* gene were single nucleotide variants; five of which were in the coding regions with one case exhibiting a single nucleotide change in the 5' untranslated region. Two variants were novel: c.1278G>A and c.1342C>G; the remaining four have been reported previously and are available on online databases (HGMD and ClinVar).

Six unique variants in *SLC25A38* were identified in 11 probands in our cohort. Six unrelated patients showed a frameshift variant—c.409dupG (p.Ala137Glyfs*16) in exon 4. Five of these cases were homozygous for the said nucleotide duplication and one was compound heterozygous. This is a novel variant that has not been described previously. Of these six cases, one was from Bangladesh and the remaining five were from the Indian states of Jharkhand, Bihar and Chhattisgarh. The single nucleotide insertion is predicted to cause a shift in the reading frame with premature chain termination. Of the remaining five unique variants identified in this gene, one was a frameshift variant (reported), three were missense/nonsense variants (one novel, two reported) and one was a splice junction variant (novel) (see figure 2).

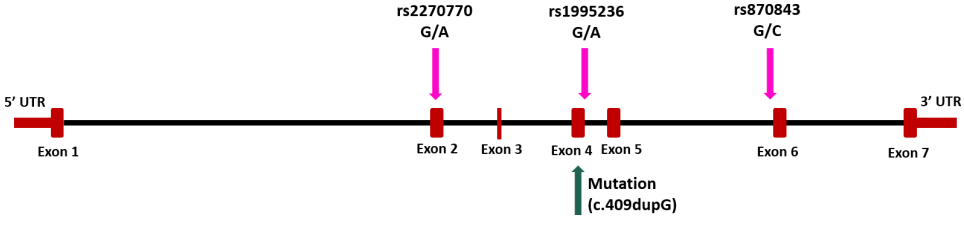


**Figure 1** Representation of the *SLC25A38* gene with the relative positions of the three polymorphisms and the common mutation (c.409dupG) used for haplotype construction. UTR, untranslated region.

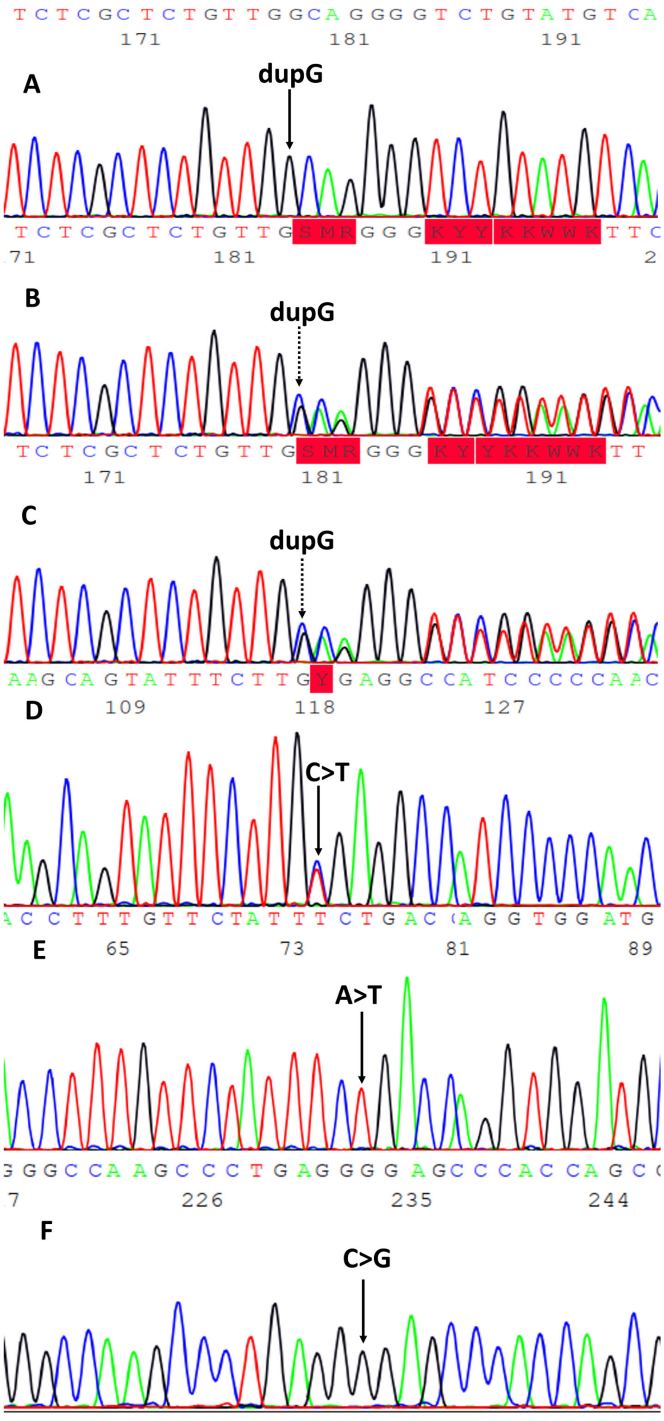


**Figure 2** Sanger sequencing with variants: (A) homozygous c.409dupG in *SLC25A38* (solid arrow) in case 16, (B) and (C) parents of above case are carriers for the same variant (dotted arrows), (D) heterozygosity for one of the mutations in *SLC25A38* in case 17 (non-sense variant) (c.349C>T) (solid arrow), (E) homozygous splice site variant in *SLC25A38* (c.626-2A>T) in case 15 (solid arrow) and (F) hemizygous missense variant in *ALAS2* (c.1342C>G) in case 6 (solid arrow).

Molecular analysis of one/both parents was possible for six patients with variants in *SLC25A38* and the maternal sample of two patients with an *ALAS2* variant. Heterozygosity for corresponding variants was confirmed in these cases. The sibling of Patient 14 who had similar clinical presentation was found to be homozygous for the same variant in *SLC25A38* (table 1).

### Haplotype construction

To establish the haplotype in the common novel variant described above, we identified three SNPs in linkage in the *SLC25A38* gene. It was observed that 16/16 (100%) mutated alleles and 1/32 (3.1%) wild type alleles showed the GGG haplotype. This strongly suggests a common founder effect for this variant ($p<0.001$) (figure 1 and table 2).

### Characterisation of the *ALAS2* promoter variant

*ALAS2* expression by RQ-PCR in the proband with promoter variant in *ALAS2* (c.-111G>A) was found to be 100-fold lower when compared with controls. This finding indicates that this promoter variant affects *ALAS2* gene expression.

### Genotype-phenotype correlation

The *ALAS2* mutated cases had significantly later presentation (median: 84 months) than the *SLC25A38* cases (median: 4 months) ($p<0.05$); with the oldest patient being diagnosed with an *ALAS2* variant at 57 years of age (Case 5). There was no significant difference between the two groups for the following parameters: haemoglobin levels at presentation, reticulocyte count and ring sideroblast percentage (figure 3). None of the patients had any characteristic features associated with syndromic sideroblastic anaemia. All patients with *SLC25A38* variants and two out of six patients with *ALAS2* variants were transfusion dependent. Three cases (patients 18, 19 and 20) did not have variants in either gene. Of these cases, one was male and the remaining two were females. All of these probands had severe symptoms and required regular transfusions. Bone marrow examination revealed 70%, 98% and 36% ring sideroblasts, respectively (table 1). Cytogenetic evaluation was performed in one of these cases, which showed a normal karyotype.

Three cases with *ALAS2* variants showed improved haemoglobin levels and/or became transfusion independent on pyridoxine treatment. The remaining cases were lost to follow-up. All patients with *SLC25A38* variants continued to require transfusions. One case (Patient 8) underwent haploidentical bone marrow transplantation but succumbed to complications. Patient 17 underwent a matched unrelated donor transplant and is currently well after a follow-up of 2 months.

## DISCUSSION

The results reported here for 17 probands with confirmed molecular diagnosis is the largest single-centre study from the Indian subcontinent. Numerous researchers have identified *ALAS2* and *SLC25A38* to be the two most common causative genes involved in CSA.[19–21] Variants in *ALAS2* contributed to 30% of cases in our study. A literature review of larger studies shows a highly variable proportion of *ALAS2* mutated cases ranging from 37% to as high as 78.9%.[1 22 23] Studies from European and Chinese cohorts have shown *ALAS2* to be the most frequently mutated gene in CSA.[19 20] Overall, we found that *SLC25A38* is more commonly mutated in our CSA cases and causative variants could be identified in most cases by screening these two genes.

*ALAS2* encodes the erythroid-specific isoform of aminolevulinic acid synthase which catalyses the first reaction in the synthesis of haem. This step involves the condensation of glycine and succinyl CoA and requires pyridoxal phosphate (PLP) as a co-factor.[14 24] Exon 9 of *ALAS2* contains the active PLP binding lysine residue and most variants have been observed from exons 5 to 11.[24 25] The missense variant identified in Case 1, p.Cys395Tyr causes the cysteine to be replaced by a larger amino acid tyrosine, which will affect PLP binding. The activity of the

**Table 1** Clinical, demographic and laboratory findings and genetic information of all cases

| Case no. | Age at presentation (months) | Sex | Place of origin State, if available (Country) | Haemoglobin at presentation (g/L) | Ring sideroblasts (%) | Reticulocyte count (%) | Serum ferritin (µg/L) | Gene | Variant identified | Prediction tools | | | | Zygosity | Variant class |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | MutationTaster | PROVEAN | PolyPhen-2 | Human Splicing Finder | | |
| 1 | 8 | Male | Kerala (India) | 20 | 20 | 1.18 | 1231 | *ALAS2* | c.1184G>A (p.Cys395Tyr)* | Disease causing | Deleterious | Possibly damaging | – | Hemizygous | Pathogenic |
| 2 | 60 | Male | Andhra Pradesh (India) | 36 | 20 | 0.65 | 1162 | *ALAS2* | c.-111G>A* | – | – | – | – | Hemizygous | Pathogenic |
| 3 | 120 | Male | Gujarat (India) | 83 | 45 | 3.45 | 1009 | *ALAS2* | c.606G>A (p.Met202Ile)* | Disease causing | Deleterious | Benign | – | Hemizygous | Likely pathogenic |
| 4 | 48 | Male | Tamil Nadu (India) | 60 | 20 | 0.55 | 1089 | *ALAS2* | c.1278G>A (p.Met426Ile)† | Disease causing | Neutral | Benign | – | Hemizygous | Likely Pathogenic |
| 5 | 684 | Male | Andhra Pradesh (India) | 57 | 20 | 2.28 | Not done | *ALAS2* | c.1354C>T (P.Arg452Cys)* | Disease causing | Deleterious | Probably damaging | – | Hemizygous | Pathogenic |
| 6 | 108 | Male | Assam (India) | 52 | 54 | 1.91 | 880.3 | *ALAS2* | c.1342C>G (p.Arg448Gly)† | Disease causing | Deleterious | Benign | – | Hemizygous | Likely Pathogenic |
| 7 | 1 | Male | (Bangladesh) | 64 | 55 | 0.19 | 1935 | *SLC25A38* | c.409dupG (p.Ala137Glyfs*16)† | Disease causing | – | – | – | Homozygous | Pathogenic |
| 8 | 1 | Male | (Nepal) | 84 | 41 | 0.29 | 1236 | *SLC25A38* | c.480dupT (p.Ile161Tyrfs*12)* | Disease causing | – | – | – | Homozygous | Pathogenic |
| 9 | 4 | Male | Jharkhand (India) | 18 | 43 | 0.46 | 1059.3 | *SLC25A38* | c.562G>C (p.Asp188His)† | Disease causing | Deleterious | Probably damaging | – | Homozygous | Likely pathogenic |
| 10 | 6 | Male | Jharkhand (India) | 40 | 49 | 2.68 | 532.2 | *SLC25A38* | c.409dupG (p.Ala137Glyfs*16)† | Disease causing | – | – | – | Homozygous | Pathogenic |
| 11 | 2.5 | Male | Jharkhand (India) | 70 | 40 | 0.24 | 4386 | *SLC25A38* | c.409dupG (p.Ala137Glyfs*16)† | Disease causing | – | – | – | Homozygous | Pathogenic |
| 12 | 3 | Male | Chhattisgarh (India) | 80 | 48 | 0.57 | 5299 | *SLC25A38* | c.409dupG (p.Ala137Glyfs*16)† | Disease causing | – | – | – | Homozygous | Pathogenic |
| 13 | 8 | Female | Jharkhand (India) | 40 | 30 | 0.49 | 395.1 | *SLC25A38* | c.562G>C (p.Asp188His)† | Disease causing | Deleterious | Probably damaging | – | Homozygous | Likely pathogenic |
| 14 | 30 | Female | Tamil Nadu (India) | 39 | 39 | 1.17 | 252.8 | *SLC25A38* | c.560G>A (p.Arg187Gln)* | Disease causing | Deleterious | Probably damaging | – | Homozygous | Likely pathogenic |
| 15 | 36 | Male | Odisha (India) | 61 | 52 | 1.68 | 3245 | *SLC25A38* | c.626-2A>T† | Disease causing | – | – | Most probably will affect splicing | Homozygous | Pathogenic |
| 16 | 0.25 | Male | Jharkhand (India) | 56 | 30 | 0.23 | 524 | *SLC25A38* | c.409dupG (p.Ala137Glyfs*16)† | Disease causing | – | – | – | Homozygous | Pathogenic |
| 17 | 5 | Male | Bihar (India) | 35 | 20 | 2.82 | Not done | *SLC25A38* | c.409dupG (p.Ala137Glyfs*16)† c.349C>T (p.Arg117*)* | Disease causing Disease causing | – – | – – | – – | Compound heterozygous | Both Pathogenic |
| 18 | 42 | Male | Karnataka (India) | 37 | 70 | 0.37 | 956 | Not detected | | | | | | | |
| 19 | 204 | Female | Meghalaya (India) | 30 | 98 | 1.9 | 1435.7 | Not detected | | | | | | | |
| 20 | 396 | Female | Andhra Pradesh (India) | 60 | 36 | 4.3 | 2990.4 | Not detected | | | | | | | |

*Previously reported.
†Novel.

**Table 2** Results of haplotype construction using three SNPs that were in linkage in the *SLC25A38* gene (rs2270770, rs1995236 and rs870843). The GGG haplotype was observed in 100% of the mutated alleles (p<0.001)

| Haplotype | Mutated alleles (n=16) | Wild type alleles (n=32) |
|---|---|---|
| GGG | 16/16 (100%) | 1/32 (3.1%) |
| AAG | 0 | 23/32 (71.8%) |
| GAG | 0 | 2/32 (6.3%) |
| GAC | 0 | 6/32 (18.8%) |

SNPs, single nucleotide polymorphisms.

mutant enzyme is expected to be salvaged at higher levels of PLP.[26] A novel amino acid substitution (p.Met426Ile) was identified in this cohort. It has been previously postulated that a similar variant, p.Met426Val can alter protein function as the branched structure of valine can affect PLP binding by interacting with the neighbouring Gly220 and Ala221 residues of the glycine-rich stretch.[26] It is possible that the present variant may have a similar effect, as isoleucine is also a branched amino acid. Another novel missense variant was also identified where the Arg448 residue is replaced by glycine. Arg448 is a surface hydrophilic residue and it has been noted that its replacement with a hydrophobic amino acid can produce sideroblastic anaemia.[26] Based on the evidence presented above, the two novel variants in *ALAS2* (Cases 4 and 6) were classified as 'likely pathogenic'. A previously reported variant in several individuals with CSA (c.606G>A) was also present in our cohort.[20 27]

The regulatory region of the *ALAS2* gene contains transcription factor binding elements. The 5' untranslated region (5'UTR) contains an iron-responsive element that regulates *ALAS2* expression by interacting with iron-responsive proteins.[28] Bekri *et al* showed that a promoter variant caused CSA in a patient with reduced *ALAS2* activity.[29] The 5'UTR variant, c.-111G>A in Case 2 has been reported previously as a variant of uncertain significance in the ClinVar database. We found that the expression of *ALAS2* in the proband with this variant was 100-fold lower, thus establishing its pathogenicity.

*SLC25A38* serves as a mitochondrial glycine transporter. Variants of this gene were found to be causative in the pathogenesis of sideroblastic anaemia by Guernsey *et al* in 2008.[3] Studies revealed that exogenous folate and glycine could restore normal haemoglobin levels in zebrafish models, but the same could not be replicated in human subjects.[30 31] In our cohort, variants in *SLC25A38* were more frequent than *ALAS2*. Five unrelated probands were homozygous and a sixth was compound heterozygous for the same novel single nucleotide duplication: c.409dupG (p.Ala137Glyfs*16). This appears to be a recurrent variant in the Indian subcontinent. We also identified that the GGG haplotype was shared by all the mutated alleles. This points to the 'founder effect' and resultant random genetic drift of the variant-associated haplotype in the population. These findings are important in the light of high incidence of consanguinity and endogamy that exists in our region. Another frameshift variant, c.480dupT seen in a Nepali patient, has been observed previously in a case with the same ethnic background.[32] A novel missense variant (c.562G>C) has been cautiously classified as 'likely pathogenic' based on it being identified in two unrelated probands (Cases 9 and 13) and other considerations as per the ACMG criteria.

A study conducted by Fouquet *et al* revealed several systemic symptoms to be associated with inherited sideroblastic anaemia.[19] Le Rouzic *et al* recognised an increased time lag between clinical and molecular diagnosis in patients with *SLC25A38* mutations.[21] However, no study has identified specific clinical features that may help in differentiating the two sets of patients. Although all laboratory parameters studied are non-discriminatory, we have established that age at presentation is a valuable clue to the underlying genetic defect.

Patients with pyridoxine or thiamine sensitive sideroblastic anaemia may benefit from pyridoxine or thiamine supplementation and may become transfusion independent. For a significant number of cases, this mode of therapy bears no effect and bone marrow transplantation may be the only curative option.[33]

Three patients (15%) in our study did not have causative variants in the two tested genes. Surprisingly, the percentage of undiagnosed cases in this study is lower than other studies conducted with more extensive gene panels.[19 22] These patients may harbour variants in other genes and require further investigations to identify them. With the arrival of next-generation sequencing, it has become possible to identify rarer genes, enabling us to understand the pathophysiology of CSA and develop potential targets for gene therapy in the future.

## Conclusion

This study represents the first case series of causative *ALAS2* and *SLC25A38* variants causing sideroblastic anaemia in the Indian

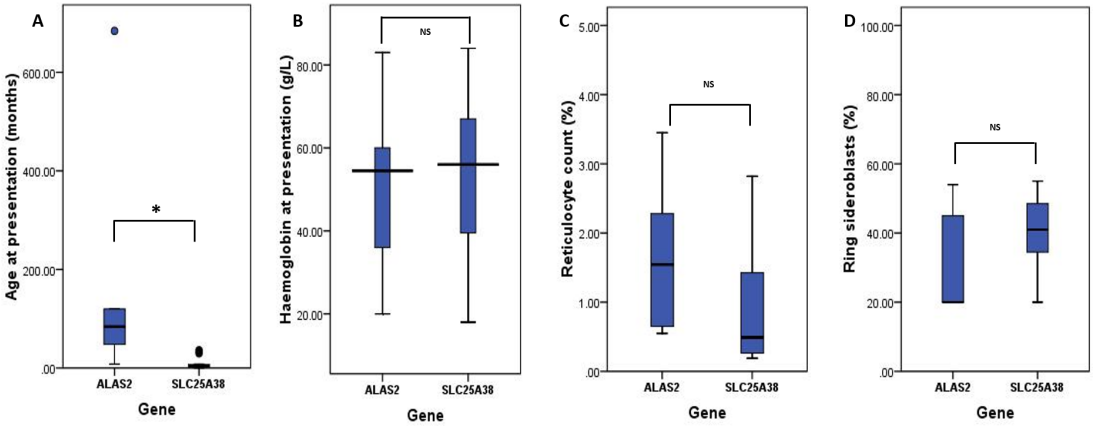


**Figure 3** Statistical analysis demonstrated the following findings between the *ALAS2* and *SLC25A38* mutated groups: (A) significant difference (p<0.05) for the age at presentation; (B), (C) and (D) no significance for haemoglobin levels at presentation, reticulocyte count and percentage of ring sideroblasts observed in the bone marrow.

subcontinent. Screening for variants in these two genes establishes diagnosis in the majority of cases of CSA in our population. We have identified a novel frameshift variant exhibiting founder effect in the *SLC25A38* gene. Inherited aetiology should always be considered in older male patients with sideroblasts in the bone marrow as *ALAS2* variants are known to present in later decades. Higher throughput techniques can help identify variants in rarer genes. Further understanding of the pathogenesis of sideroblast formation can help in shaping newer therapies for these patients.

## Take home messages

- The study reports that *SLC25A38* gene is more commonly implicated in patients with inherited sideroblastic anaemia from the Indian subcontinent.
- A common frameshift variant in *SLC25A38* with founder effect is present in most cases.
- Patients with *ALAS2* variants may present at later age.

**Handling editor** Mary Frances McMullin.

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**Acknowledgements** The authors are grateful to the technical staff for their support.

**Contributors** ESE and NR conceived the study. NR, ES, RA and SS designed and performed the tests. UK, AK, FNA and BG provided clinical input. NR wrote the manuscript. ESE, UK, RVS and BG reviewed the manuscript.

**Funding** The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

**Competing interests** None declared.

**Patient consent for publication** Not required.

**Ethics approval** The study was approved by the Institutional Review Board.

**Provenance and peer review** Not commissioned; internally peer reviewed.

**Data availability statement** Data sets generated during the study are available in the published article and supplementary data or available at request from the corresponding author.

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