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# The molecular biology and pyridoxine responsiveness of X-linked sideroblastic anaemia

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**ABSTRACT**

**Pyridoxine-responsive, X-linked sideroblastic anaemia (XLSA) has been shown to be caused by missense mutations in the erythroid-specific ALA synthase gene, ALAS2. These are scattered widely across the part of the gene encoding the catalytic domain and in half the cases affect residues conserved throughout evolution. Only a loose correlation has been found between the *in vitro* kinetics and stability of the catalytic activity of the recombinant variant enzymes and the *in vivo* severity and pyridoxine-responsiveness of the anaemia. Enhanced instability in the absence of pyridoxal phosphate (PLP) or decreased PLP and substrate binding have been noted. A detailed explanation of the anaemia and its response to pyridoxine, however, requires greater insight into the structure-function relationships of this protein than we have at present. Knowledge of its tertiary structure and further knowledge of intracellular factors which impinge on the ability of normal and variant ALAS2 to contribute to haemoglobin production are also required. Mutations in the same gene which affect mitochondrial processing, terminate translation prematurely, or are thought to abolish function altogether cause an XLSA that is refractory to treatment with pyridoxine. A major complication of this disorder is its accompanying increased iron absorption and iron overload which occurs in patients and female heterozygotes. Mutation detection enables the early diagnosis of those affected, targeted education of families, early treatment with pyridoxine and prevention of iron overload. It also allows for a distinction to be made between late-onset variants of this condition and the more insidious refractory anaemia with ring sideroblasts. The next few years of investigation should be illuminating as tools now exist to study all aspects of this protein from the gene to the mitochondrial matrix.**

*Key words: sideroblastic anemia, pyridoxine*

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The haemoglobin produced by the continuous and regenerative process of erythroid differentiation requires a supply to erythroid precursor cells of about 24 mg iron each day.[1] A corresponding amount of protoporphyrin IX must be available for incorporation of iron to make haem within erythroblast mitochondria. This is achieved, upon differentiation, by an erythroblast-specific up-regulation of the enzymes of the haem synthesis pathway.[2] Most of the haem biosynthesis genes are transcriptionally activated by several erythroid-specific factors operating on promoter elements distinct from those controlling these same genes in non-erythroid tissues. Interestingly, the first, and possibly rate-limiting, enzyme of the pathway, 5-aminolevulinate synthase (ALAS), is expressed from a different gene to that used to regulate non-erythroid haem biosynthesis. The housekeeping gene, expressed at low levels in all tissues, is designated ALAS1 and the erythroid-specific gene, expressed at high levels, is designated ALAS2.

The ALAS1 and ALAS2 isozymes share 72% amino acid identity in the central and C-terminal two thirds of their sequence and are structurally very similar. Both are dimers consisting of two identical subunits catalyzing the synthesis of 5-aminolevulinic acid, the first intermediate of the haem synthesis pathway, from substrates glycine and succinyl CoA. Consistent with the fact that succinyl CoA is formed only within mitochondria, ALAS is ultimately translocated into the mitochondrial matrix, possibly associated with the inner membrane.[3] Recent evidence suggests that the catalytic site is located at the subunit interface[4] and catalytic activity is completely dependent upon the presence of pyridoxal 5'-phosphate as cofactor.[3]

The key role of ALAS2 in erythroid heme biosynthesis is highlighted by the iron-loading anemia resulting from mutations compromising its activity or stability. X-linked sideroblastic anaemia (XLSA) was first described in 1945 by Thomas Cooley[5] who reported the clinical and haematological features of two brothers from a large family which over multiple prior generations had experienced the early deaths of affected males. Pyridoxine-responsive anaemia was described subsequently (1956)

by Harris *et al.*[6], as well as in Cooley's original two patients[7] and over the following 8 years more than 70 similar cases were reported.[8] Of note was the predominance of males (80%) among those affected and the presence of a red cell mosaicism (small, hypochromic cells as well as normal red cells) in some of their mothers, female siblings and female relatives on their mother's side. Such a sex-linked form of inheritance indicated an X chromosomal location for the causative gene.

The diagnostic features of note in XLSA were (a) small, hypochromic red cells in the absence of iron deficiency (Figure 1), (b) a non-haemolytic anaemia, (c) a hypererythroid marrow, and (d) the presence in the marrow of significant numbers of ringed sideroblasts-erythroblasts with excess iron in the mitochondria arranged as a rings around the nuclei. There was no consistent pattern of involvement of white cells and platelets. These features suggested a localised abnormality of haem production with the iron taken up by the erythroblast accumulating in the mitochondrion when faced with either a deficit of, or an inability to pair with protoporphyrin IX. Low or normal levels of free red cell protoporphyrin indicated the former.[8] For an extensive treatment of the haematological aspects of this condition, the reader is referred to the review of Professor Sylvia Bottomley.[9]

The sometimes marked haematological response to pyridoxine, the similarity to pyridoxine deficiency-induced anaemia in animals[10] and the incidence (albeit rare) of secondary sideroblastic anaemia with similar features to the X-linked form after treatment with pyridoxine antagonists such as isoniazid[11] focused attention on an abnormality in the pyridoxal 5'-phosphate (PLP)-dependent ALAS. This was supported by the low levels of enzyme activity often measured *in vitro* in erythroblasts from affected patients.[12,13] However, whether or not this was a true deficiency or a down regulation occurring as a secondary effect of another primary lesion could not be discerned. Furthermore, there was no explanation at that time for the limitation of the defect to only the erythroid cells, or for the X-linked mode of inheritance.

The existence of an erythroid-specific enzyme was suspected from the studies of Bishop (1981)[14] and Watanabe *et al*. (1983)[15] showing biochemical, physical and immunological differences between the proteins isolated from adult liver and erythroid tissue of both guinea pig and chicken. In 1985, Yamamoto[16] isolated a partial ALAS cDNA clone from a reticulocyte expression library which identified an erythroid-specific mRNA. After considerable controversy regarding the existence of one versus two genes for this enzyme, Riddle *et al.* (1989)[17] showed conclusively that there were different hepatic and erythroid ALAS mRNAs in the chicken. The human erythroid ALAS mRNA was subsequently isolated, sequenced and the gene mapped to Xp11.12.[18-22] This gene (ALAS2) was thus firmly established as the best candidate for X-linked sideroblastic anaemia.

ALAS2 was shown to be the gene defective in XLSA by the discovery of an individual with a point mutation in exon 9 of this X-linked gene resulting in the substitution of isoleucine 476 by asparagine.[23] *In vitro* enzymatic assay of the recombinant normal and mutant enzymes revealed a 99% loss of activity due to this mutation.[23] Subsequently, 21 different ALAS2 point mutations have been described in XLSA patients.

## The structure of the ALAS2 gene

The human ALAS2 gene spans about 22 kb and consists of 11 exons of varying sizes. The promoter sequence elements are typically erythroid with cognate binding sites for the transcription factors GATA-1, NF-E2 and CACCC (EKLF).[19,24 and Bishop, unpublished] These three factors also play a crucial role in the erythroid-specific activation and up-regulation of globin genes. Also present in the ALAS2 promoter are



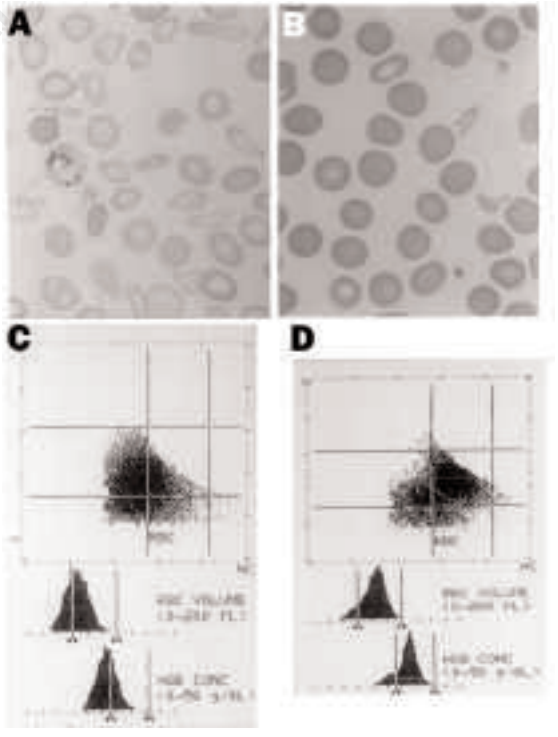


**Figure 1. The blood film (A,B) and red cell appearances (C,D) of a male patient with pyridoxine-responsive X-linked sideroblastic anaemia (A,C) and his sister (B,D). The scattergrams and histograms (C,D) were obtained using the Bayer Technicon H3 automatic cell analyser.**

TATA and CCAAT boxes and a thyroid response element just immediately prior to the transcription initiation site.

The intron/exon structure of the human ALAS2 gene is very similar to that of the mouse and the chicken with exons 6 to 10 identical in size and boundaries.[19,25,26] Exon 11 differs only in the length of the 3' untranslated region while less sequence homology and variation in both exon size and junction sequence occur in the first 4 exons and the 5' end of exon 5. Significant alternative splicing which resulted in the deletion of exon 4 was seen in human erythroid tissue.[19] Additionally, in the mouse, 15% of the ALAS2 mRNA was alternatively spliced by deletion of the first 45 nucleotides of exon 3.[26] Because of the lack of sequence conservation, the toleration of alternative splicing in exons 1-4 and the fact that bacterial ALAS lacks this region, it has been proposed that exons 5-11 constitute the ancestral catalytically competent core of the enzyme.[17,26,27]

## Regulation of gene expression

### *Transcriptional up-regulation of ALAS2*

ALAS2 gene expression is tissue-specific, occurring normally only in adult and foetal erythroid tissue. Studies with cultured erythroid precursor cells have established that numerous cytokines and hormones including erythropoietin are required for terminal differentiation to erythrocytes.[28] Some of the necessary transcription factors required in erythroid cells to support this are GATA-1, EKLF, and NF-E2. Putative binding sites for these factors are found in the ALAS2 promoter (see above) but the precise pathway between factors in the environment and up-regulation of this gene is yet to be defined. In cultured erythroleukaemia cells, terminal differentiation can often be chemically induced by such agents as dimethyl sulphoxide (DMSO) in murine MEL cells[29] and by hemin or butyrate in human K562 cells.[30] Studies of DNAase hypersensitivity (HS) of the ALAS2 gene in both DMSO-induced and uninduced mouse erythroleukaemia cells identified 5 HS sites that were absent from a non-erythroid fibroblast cell line.[25] In these cells, transcriptional activation of ALAS2 may be due to increased levels of transcription factors acting at the preformed HS sites. Whether or not homologous sites exist in the human ALAS2 gene, if they are indeed erythroid-specific and how they become formed during the process of erythroid commitment remains to be seen.

The constitutively expressed housekeeping gene, ALAS1 is also transcribed in erythroid cells, albeit at extremely low levels. Although there is evidence for a modest downregulation of ALAS1 in certain cell lines induced to differentiate along the erythroid pathway this is not complete and is not a prerequisite for up-regulation of the ALAS2.[31-34]

### *RNA processing*

Alternative splicing of the primary transcript has been noted in both human and murine ALAS2 (see above) but there is no evidence for this being a significant factor in the control of gene expression.[24] In human erythroid cells, both splicing products were observed in proerythrocytes and reticulocytes as well as in several erythroleukaemia cell lines.[24] While the PCR method used was not quantitative, the amounts in all cases were relatively constant and did not change with the stage of differentiation. In both species, the deletions are in frame and should lead to protein structural heterogeneity. Since the catalytically active core of the enzyme appears to reside in the C-terminal part of the protein coded for by exons 5 to 11, these deletions may not affect activity and/or stability but no studies have addressed these questions.

### *Regulation of translation by iron*

The 5' untranslated region of the murine ALAS2 transcript was shown to contain a sequence similar to the iron-responsive element (IRE) sequence found in rat and human ferritin.[32] Subsequently, this sequence motif was identified in nucleotides 2 to 46 of the human ALAS2 transcript covering all of exon 1 and the first 10 nucleotides of exon 2, just prior to the start AUG.[19,35] The IRE sequence is able to base-pair internally to form a secondary structure resembling a hairpin with a 6 nucleotide unpaired loop. IRE binding proteins were shown to complex functionally with this ALAS2 region and confer iron-mediated control of translation in MEL cells[36] and in a cell-free translation system using purified IRE binding protein.[37] The IRE-binding protein is able to bind to this hairpin structure and inhibit translation only when it has become depleted of iron and thereby functions to coordinate iron supply with heme synthesis. The discovery of the ALAS2 IRE represents a major advance in the understanding of the regulation of erythroid heme biosynthesis.

### *Regulation of transport into the mitochondrial matrix*

Translocation from the cytosol to the mitochondrion for nuclear DNA-encoded proteins differs for the particular protein but in general requires ATP, an electrochemical potential across the mitochondrial membrane and involves many steps and interactions.[38] The protein has to be kept unfolded to enter the mitochondrion and may then need help from the mitochondrial matrix chaperonins to become correctly folded when it has reached its destination. A leader peptide is probably required to direct the protein to the mitochondrial matrix but other sequences may be involved in the initial binding to a mitochondrial surface receptor. Specific interaction with a peptidase within the mitochondrion is necessary to remove the leader peptide and

allow it to take up its final conformation and localization.

The translation start codon of the human ALAS2 transcript begins with the 16th nucleotide of exon 2 and encodes a leader peptide for targeting to the mitochondrial matrix which is possibly either 49 amino acid residues (by homology to the known cleavage site of rat ALAS1)[39] or 78 residues (by homology to the cleavage site of the ornithine transcarbamylase leader peptide).[40] The termination codon occurs within exon 11 giving rise to a 65 kDa precursor protein of 587 amino acids.[18,19] The polyadenylation signal AATAAA occurs 105 nucleotides after the termination codon. The cleavage point for the leader peptide in human ALAS2 has not been determined experimentally, but recent immunochemical studies of erythroblast mitochondrial ALAS2[41] have identified a major protein band of about 56 kDa which would be consistent with enzymatic cleavage after residue 78.

The leader peptide may confer a feed-back regulatory role on erythroid ALAS activity by a haem-mediated inhibition of transport into the mitochondria.[42] The enzyme half-life within the mitochondria is only one hour therefore such an inhibition would be an effective means of control. An important study by Lathrop and Timko[43] demonstrated that the haem-mediated inhibition of translocation is conferred by repeated Cys-Pro amino acid motifs in the leader peptide of murine ALAS2.[43] The functional role of these residues was evaluated in cell-free mitochondrial studies showing that either of the first two most N-terminal motifs were sufficient to confer inhibition of translocation by 10-25 μM haem. Only when both cysteines were mutated to serines was haem unable to block import. A preliminary report on import of rat ALAS2 into quail fibroblast mitochondria, however, showed no sensitivity to the presence of haem.[44] Thus, the extent to which these conserved motifs play a role in the normal physiological regulation of human ALAS2 needs clarification.

That ALAS2 is a mitochondrial enzyme opens up a new dimension on the regulation of erythroid differentiation. The mitochondrial space is a significant proportion of the erythroblast and yet little is known about it. There are thought to be many post translational events involved in getting ALAS2 from the ribosome to the mitochondrial matrix. These are all points at which regulatory factors may interact and are therefore all points at which gene mutations may interfere and cause pathological changes.

### *X-chromosome inactivation in women*

A further level of control over gene expression occurs in women due to random X-chromosomal inactivation.[45] Early in embryogenesis, each cell will randomly inactivate one of its two parental X chromosomes (Lyonization). Thereafter, the same parental chromosome will remain inactivated in all the cell's progeny. There is a normal distribution of inactivation in the female population with the majority of individuals near 50:50 for their parental X chromosomes while a small but significant percentage will be highly skewed. Thus the phenotype of an XLSA heterozygote ranges from severely affected to normal.

## Structure and function relationships for the ALAS2 protein

Studies of ALAS2 structure and function required its purification and *in vitro* measurement of its activity. This was first achieved by large scale isolation of the protein from erythroid tissue. Difficulties included the small amounts of protein present, considerable proteolytic degradation and insolubility. More recently, recombinant DNA technology has been used. High-level, prokaryotic expression of native or fusion-protein constructs for human or murine ALAS2 can be purified easily using affinity or classic chromatographic methods.[46,47] Kinetic properties of the recombinant and non-recombinant native proteins were very similar. The $K_m$ for glycine is high, in the millimolar range while the $K_m$ for succinyl CoA is in the micromolar range as is the binding constant for pyridoxal 5'-phosphate.[46] High specific activities have been obtained.[4,48] Site-directed mutagenesis has enabled the production of variant proteins corresponding to the mutations found in patients with XLSA whose structure and function can then be compared (see below).

A crystal structure for the protein does not yet exist but a hypothetical three dimensional picture is being built up by the use of computer models for protein structure prediction and comparison of the amino acid sequence with those of the same protein from different eukaryotic and prokaryotic species and also with those from other pyridoxal-phosphate dependent enzymes, some of which have known tertiary structures.[49,50] By identifying conserved motifs of amino acid sequence and noting their position/function in some of these other proteins, hypotheses regarding their function in ALAS2 can be formulated. These are then tested using recombinant DNA technology to replace individual amino acids.

Recombinant proteins with different single amino acid substitutions thought to be within the active site and which had no catalytic activity when expressed as homodimers were found to produce functional protein with about 25% of normal activity when coexpressed and isolated as heterodimers.[4] These studies suggested that the two subunits of ALAS2 are in contact across their catalytic sites. Examination of the murine ALAS2 peptide to which PLP was bound irreversibly after sodium borohydride reduction showed that lysine 313, homologous to lysine 391 in human ALAS2, coded for at

the beginning of exon 9, formed a Schiff base with PLP.[51] Somewhat surprisingly, substitution of this lysine by another amino acid does not prevent the binding of PLP or the substrate glycine, however the enzyme is catalytically inactive.[52] It was therefore suggested that the lysine is directly involved in the catalytic reaction, probably at the level of deprotonation of the subsequently bound glycine.[52] Among those other parts of the molecule thought to be involved in binding PLP is a glycine-rich region (GAGAGG) coded for by exon 6.[53] This is thought to be important in binding the phosphate part of PLP and is probably also involved in substrate binding in some way since alterations of these residues decrease binding of substrates as well as PLP.[53] The equivalent residue in mouse ALAS2 to arginine 227, just adjacent to this glycine-rich region has also been shown by substitution to be essential for catalytic activity however the precise mechanism of this involvement awaits further study.[53]

Comparison with other PLP binding enzymes permits categorisation of the ALAS protein as a type II aminotransferase of fold type I.[50] Analysis of the homologous sequences suggests that the aspartic acid 357 and arginine 517 are important in binding the pyridoxal ring nitrogen.[54] It also suggests that the hydrophobic β sheet region just before the Schiff base-forming lysine, could be involved in the non-covalent binding of the pyridoxal ring.[50] It will be of interest to compare these predictions to the actual structure, if and when a three-dimensional structure is determined by X-ray crystallography.

## Genotype-phenotype in inherited disorders of ALAS2

Two groups, one in New York and the other in Oklahoma, have been responsible for demonstrating the relationship between mutations in the erythroid-specific ALAS2 and XLSA in the majority of cases studied so far. There are now over 22 mutations described in more than 30 kindreds.[23,47,48,55-60] These are summarised in Table 1 and Figure 2 indicates how they are clustered with respect to the gene structure, all being found in exons 5 to 11. Although most patients studied have been male it is notable that almost 25%[8] of the patients (data not shown) are female.



**Table 1. The nature of the predicted amino acid substitutions in XLSA caused by mutation in the ALAS2 gene and the pyridoxine responsiveness of the associated anaemia.**

| *Pyridoxine responsiveness* | *Mutation* | *Predicted amino acid substitution* | *Secondary structure prediction** | *Nature of wild-type amino acid* | *Conservation°* | *Nature of substituted amino acid* | *References* |
|---|---|---|---|---|---|---|---|
| *refractory* | | | | | | | |
| | 1601 **C**GC→**T**GC | R517C (*F*) | β sheet | large, basic | 100% | small, polar, SH | Bottomley# |
| | 731 **C**GC→**T**GC | R227C (*F*) | β sheet | large, basic | 100% | small, polar, SH | Bottomley# |
| | 662 **C**GA→**T**GA | R204stop(*F*) | *not relevant* | *not relevant* | – | *not relevant* | Bottomley# |
| | 621 G**A**T→G**T**T | D190V | none | small, acidic | <40% | medium, non-polar | 41 |
| | 561 C**G**C→C**A**C | R170H (*F*) | α helix | large, basic | 100% | medium, basic | Bottomley# |
| *partial* | | | | | | | |
| | 1731 **A**GT→**G**GT | S568G | α helix | small, polar | >75% | small, polar | Bottomley# |
| | 1574 **A**CT→**T**CT | T508S | loop | small, polar | 100% | small, polar | Bottomley# |
| | 1407 C**G**C→C**A**C | R452H | α helix | large, basic | 50% | medium, basic | 57,59 |
| | 1406 **C**GC→**T**GC | R452C | α helix | large, basic | 50% | small, polar,-SH | 57 |
| | 1406 **C**GC→**A**GC | R452S | α helix | large, basic | 50% | small, polar | 57 |
| | 1395 C**G**A→C**A**A | R448Q | α helix | large, basic | 50% | medium, polar | 57 |
| | 1299 G**G**C→G**A**C | G416D(*F*) | none | small, polar | 100% | small, acidic | 57 |
| | 1283 **C**GC→**T**GC | R411C | α helix | large, basic | 100% | small, polar, -SH | 57 |
| | 647 **T**AC→**C**AC | Y199H | none | large, polar, aromatic | 100% | medium, basic | 58 |
| | 560 **C**GC→**A**GC | R170S | α helix | large, basic | 100% | small, polar | Bottomley# |
| | 547 TT**C**→TT**A** | F165L | α helix | large, non-polar, aromatic | > 90% | medium, non-polar | 47 |
| *considerable* | | | | | | | |
| | 1479 A**T**C→A**A**C | I476N | β sheet | medium, non-polar | < 50% | medium, polar | 23 |
| | 1331 **A**TG→**G**TG | M426V | α helix | medium, non-polar | 40% | small, non-polar | 41 |
| | 1215 A**C**T→A**G**T | T388S | β sheet end | small, polar | 100% | small, polar | 55 |
| | 923 **G**GT→**A**GT | G291S | α helix | small, polar | 100% | small, polar | 56 |
| *complete* | | | | | | | |
| | 947 **A**AG→**C**AG | K299Q | loop | large, basic | 70-80% | medium, polar | 48 |
| | 566 **G**CT→**A**CT | A172T(*F*) | α helix end | small, non-polar | 70-73% | small, polar | 48 |

**This is calculated using the PHD program provided by EMBL, Heidelberg;[85,86] °The percentage of times this position is occupied by the same amino acid as that found in human ALAS2 when all known ALAS sequences are aligned;[87] #Bottomley, 1997, personal communication; (F) indicates where female probands only have been described.*

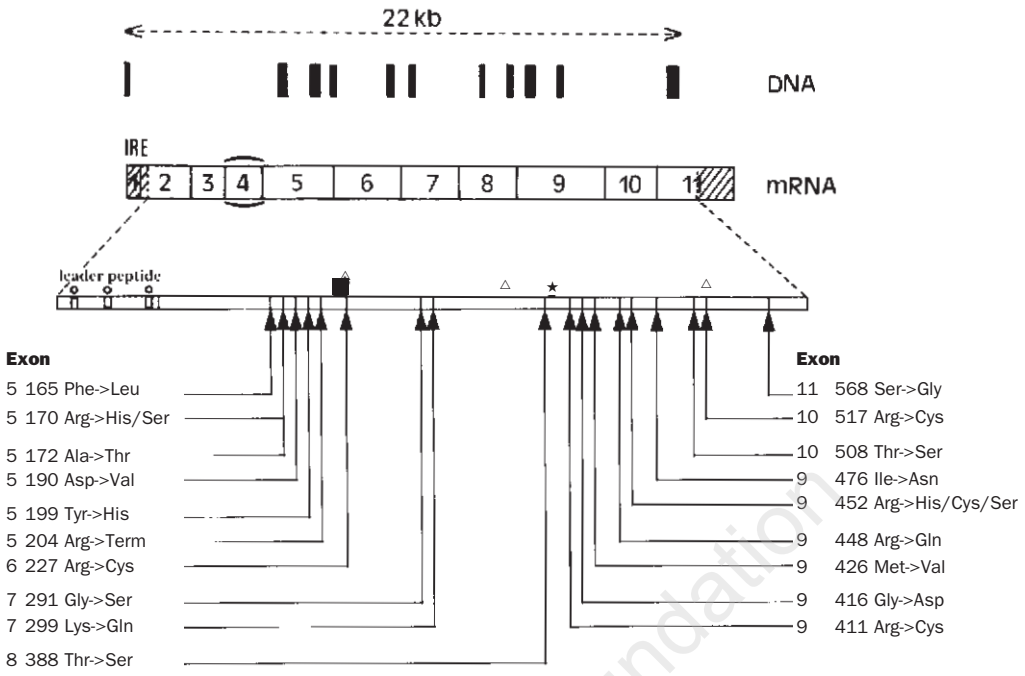


**Figure 2. The structure of the erythroid ALAS gene (ALAS2), processed RNA and protein showing (a) motifs and amino acids identified as being of essential/important function and (b) the position of the predicted amino acid replacements for the mutations observed in XLSA. This is not drawn strictly to scale. The cleavage point for the leader peptide has been shown as residue 78 but this has yet to be substantiated. ⨯ Untranslated RNA; ⌒ Alternative splicing may remove this exon; IRE=iron responsive element; ° = Cys-Pro/haem responsive motif; ■ = glycine-rich region-the putative phosphate binding site; △ key amino acid residues (from left to right: arginine 227, aspartic acid 357 and arginine 517); ★ lysine 391 which forms a Schiff base with the pyridoxal phosphate aldehyde group.**



### *Types of mutations found*

To date, all but one of the mutations are missense. They have each arisen from a single base change in the DNA leading to the substitution of a single amino acid in the protein. Several changes described in patients with XLSA have affected amino acid residues which are of such importance as to have been conserved throughout evolution in the proteins of many different species. One mutation is nonsense, replacing an amino acid codon in the RNA with a stop codon (R204Ter, Bottomley, personal communication, 1997). This leads to premature termination of translation of the protein and a nonfunctional truncation product.

### *Those parts of the protein affected by the mutations*

The missense mutations so far described have been scattered across the core functional part of the protein (that encoded by exons 5 to 11). Only two have led to the substitution of an amino acid which has been identified as having a specific and important function (arginines 227 and 517)[50,53,54] and one (threonine 388) is within the putative non-covalent binding site for the PLP pyridoxal ring.[50]

### *The association between mutation and pyridoxine responsiveness*

The responsiveness of patients with XLSA to oral pyridoxine supplementation varies considerably. Responsiveness is probably best measured as the degree to which haemoglobin levels and/or red cell indices can be normalised rather than the amount by which the haemoglobin has increased since the latter may depend upon the time at which the anaemia was discovered, the intrinsic severity of the defect and possible secondary causes such as iron toxicity. The response may be absent, partial or complete.

### *ALAS2 mutations leading to a pyridoxine-refractory sideroblastic anaemia*

Five out of the 22 mutations listed in Table 1 have been found in patients whose anaemia has not responded to pyridoxine. The pyridoxine responsiveness observed with other mutations is therefore specifically related to the nature and position of the amino acid substitution in ALAS2 since there was no observed general response to pyridoxine supplementation in the face of a complete ALAS2 deficiency.

For the mutation which results in an inactive trun-

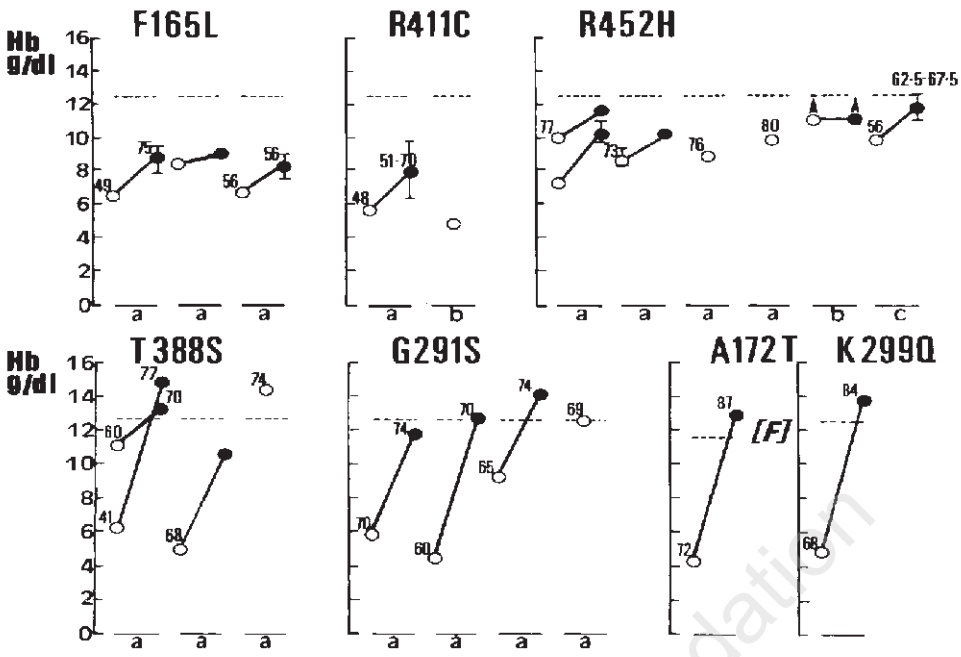


**Figure 3. The variability of the severity of anaemia and response to treatment in pyridoxine-responsive XLSA. Published data for some of the mutations which have been discussed particularly in the text are shown. Where several values are known the mean and range are given. a,b and c indicate different kindreds.**
**○: At the time of diagnosis; ●: On pyridoxine treatment; - - - lower limit of the normal range. The values included above the data points are those for the MCV in fl where they are known. N.B. The value of the MCV will vary from machine to machine depending upon the technology and calculations used for its measurement. They cannot therefore easily be compared from one study to another but have been included here to give some idea of the sort of changes that occur in an individual patient with pyridoxine treatment. Despite the technology used the lower limit of the normal range usually lies between 80-85 fl.**
***[F]* indicates a female patient.**

cated protein (R204Ter) no clinical response to pyridoxine has been observed (Bottomley, personal communication, 1997).

Three out of the four remaining mutations affect residues which have been conserved throughout evolution (arginines 517, 227 and 170) (Bottomley, personal communication, 1997). Furthermore, the equivalent residue to arginine 227 in mouse ALAS2 has been shown *in vitro* to be essential for function (discussed above).[53] By homology, arginine 517 was also deemed to be of an essential nature.[50] That these three mutations have been found only in female heterozygotes who will have varying proportions of normal and abnormal red cells makes genotype-phenotype relationships more difficult to determine without full information. It may be that these mutations cannot be tolerated in male hemizygotes[61] (this is likely to be the case for the non-functional 227 and 517 substitutions) but it is also true to say that the clinical picture regarding pyridoxine-responsiveness may be obscured by the presence of normal red cells.

The final mutation in this group was found in a male patient and predicts the replacement of aspartic acid 190 by valine.[41] This replacement is associated with quite a difference in biophysical properties in spite of the fact that this amino acid varies quite considerably anyway (glutamine, glutamic acid, serine, lysine, arginine) amongst the normal ALAS1 and ALAS2 proteins from different species (see Table 1). *In vitro* enzyme activity of the isolated recombinant enzyme was normal as was the rsponse to PLP (Table 2) and yet patient erythroblast ALAS activity was very low. The cause of the decreased erythroblast ALAS activity (and, presumably, of the associated moderate anaemia) was found to be a diminished amount and abnormal size of immunochemically detected ALAS2 protein within erythroblast mitochondria. This was proposed to be due to an abnormality of mitochondrial import and/or processing that could not be alleviated by increased PLP.[41]

### *ALAS2 mutations leading to pyridoxine-responsive XLSA*

Amongst the mutations associated with a haematological response to pyridoxine there is no obvious relationship between the mutation and the clinical response. These are scattered across the gene affecting exons 5, 7, 8, 9, 10 and 11. The associated anaemia can vary in severity as can the extent of the response. Figure 3 shows examples of different

haematological responses from patients with different mutations. Only responses for male patients are shown except in the one instance where such a severe anaemia occurred that much contribution from the normal allele can be discounted. More complicated cases amongst these kindreds who had been splenectomised or who showed evidence of coexisting vitamin B12 or folate deficiencies are omitted.

Pyridoxal phosphate binding to the enzyme is crucial for its stability, maintaining a conformation optimal for substrate binding and product release, and for the catalytic activity of the enzyme. Decreasing cofactor binding might have an effect on all these functions in a manner reversible to the extent to which the enzyme can eventually be saturated again, regain its active conformation and carry out its catalytic reaction.

Additional factors likely to affect the clinical outcome will include the degree to which an enzyme with low activity can be compensated for by increased amount within the mitochondrion and to which a low amount of enzyme can be compensated for by increased specific activity.

Preliminary *in vitro* studies of recombinant mutant enzymes cannot necessarily be compared one with another because of the differences involved in fusion versus non-fusion constructs, uncertainty in the nature of the mature N-terminus in human mitochondrial ALAS2 and differences in the degree of purification as the methodology evolves.[47-49,57] With the currently more standardised conditions, greater understanding of the solubility and stability of the recombinant product and the ability of numerous laboratories to study and compare a large number of mutant proteins, some greater understanding of how the *in vitro* activity relates to clinical outcome is emerging.

With the above caveat in mind, some comments can be made concerning the seven associated with pirydoxine responsiveness out of eight recombinant enzymes studied so far (Table 2).

**Table 2. The *in vitro* activity of recombinant mutant ALAS2 enzymes expressed as a percentage wild-type enzyme activity with and without the addition of pyridoxal phosphate.**

| *Amino acid substitution* | *Activity compared to wild type in the absence of PLP* | *Activity compared to wild type in the presence of PLP* | *References* |
|---|---|---|---|
| F165L | 13% | 26% | 47 |
| A172T | 125% | 138% | 48 |
| D190V | 100% | 100% | 41 |
| G291S | <7% | approx 50% | 56 |
| K299Q | 136% | 151% | 48 |
| T388S | approx. 52% | approx 60% | 55 |
| I476N | absent | <1% | 23 |
| M426V | low | not given | 41 |

Two variant enzymes (lysine 299 to glutamine:K299Q and alanine 172 to threonine:A172T) associated with the most complete clinical remission with pyridoxine in two elderly patients (see further discussion below) showed greater than normal enzyme activity but demonstrated a drop in activity over time in the absence of added PLP caused by increased thermolability.[48] In these cases, therefore, pyridoxal phosphate (derived from pyridoxine supplements) is apparently required to stabilise a functionally adequate protein. The cause of the high specific activity and the extent of its contribution to the pyridoxine-supported full haemoglobinization of the patient's red cells remain uncertain.

The other five enzymes (phenylalanine 165 to leucine:F165L, glycine 291 to serine:G291S, threonine 388 to serine:T388S, isoleucine 476 to asparagine:I476N and methionine 426 to valine M426V) have decreased *in vitro* activity in the absence of PLP with variable but incomplete responses to PLP in the four for which there is data. This is consistent with their association with XLSA that is only partially responsive to pyridoxine.[23,41,47,56]

## Age of onset of clinically detected XLSA

A feature of pyridoxine-responsive XLSA is the variable age of its onset highlighted in a report recently of two patients, one male and one female, who first developed their anaemia (Hb 4.9, 4.3) when they were 77 and 81 yrs old.[48] This late onset form of XLSA was found to be quite remarkably reversed by pyridoxine despite the fact that the anaemia which had developed was severe and extremely hypochromic (Figure 3: K299Q and A172T). Decreased levels of plasma pyridoxine occur in elderly people due to decreased intake and/or age-related alterations in pyridoxine metabolism.[62] It is thought that these somewhat unstable enzymes (see paragraph above) could have retained sufficient active protein for adequate enzyme activity until PLP available to the enzyme dropped below a critical level which then triggered a vicious cycle of loss through inactivation, proteolysis and iron overload. Other age-related phenomena such as decreased respiration[63] leading to decreased ability to transport and assemble the enzyme and, in women, increased skewing of X-chromosome inactivation,[64,65] may also play a part. Clearly these patients were not anaemic at birth and although the precipitant anaemia was severe this disorder could only be described as a mild one due to its being eminently treatable by pyridoxine.

Contrast this to the findings in the family originally described by Cooley.[4] Most of the boys affected were born anaemic with hypochromic red cells

and almost half of them died before 1 year of age. Pyridoxine responsiveness was only partial and increased the haemoglobin level to little more than 9 g/dL.[7,47] The disorder caused by this mutation is consistently severe. Only with modern diagnosis and management are the affected males living to middle age or beyond.

Although somewhat later in its onset (8 yr,10 yr) another severe disorder is that caused by the arginine 411 to cysteine (R411C) substitution. This has been shown to be associated with a similar degree of anaemia, early age of onset and complications of iron overload in two unrelated patients from different parts of Europe.[66]

The replacement of arginine 452 by a histidine (R452H) has been observed by three different groups[57,59] in adults (24 yr-38 yr) and appears to have a consistently mild outcome although the pyridoxine response is very small and only partial.[57,67] It is notable, however, that one patient presented at a later date with a more severe anaemia (Figure 3, R452H, 1st case). Such a variable penetrance for pyridoxine-responsive XLSA has been reported on several occasions[55,56,67] and is shown very nicely in the paper from Prades *et al.*[56] summarised in Table 3 which described several male family members of varying ages (from 16 to 48 yr) who had inherited the same mutation but who showed quite different degrees of anaemia. These disorders of intermediate severity highlight the involvement of factors other than the mutation, gender and age in the severity of the anaemia. The degree of iron loading in particular appears to play a major role.[47,56]

## Iron overload in pyridoxine-responsive XLSA

Although the anaemia of pyridoxine-responsive XLSA can be treated, the main complication of this disorder is iron overload. The haem synthesis deficiency leads to ineffective erythropoiesis and an expanded erythroblast compartment with its large demand for iron. Despite the high rate of iron delivery to the tissue, inadequate haem synthesis prevents the inhibition of its uptake by the usual feedback mechanism.[68,69] The urgency of the expanded erythropoietic demand for iron outweighs any control over dietary iron absorption exerted by body iron stores and increased iron absorption from the gut persists at an innappropriately high level. Transferrin saturation with iron increases, iron delivery to the tissues is increased and exceeds their storage capacity, serum ferritin increases in an age-dependent manner and, without prevention, diabetes, liver and heart failure result.

Iron overload from increased iron absorption alone can be present even by adolescence,[58,66] in some cases exacerbated by inappropriately prescribed iron supplements or the incidental intake of iron with fortified foods or multivitamin preparations.

**Table 3. The variable age of onset of pyridoxine-responsive anaemia, and iron stores, in members of a family with the same mutation in the ALAS2 gene. This data has been reproduced from the paper by Prades *et al.*, 1996,[56] with permission.**

| *Sex* | *Age (yr)* | *Serum ferritin (µg/L)* | *Without pyridoxine Hbg/dL* | *With pyridoxine Hbg/dL* |
|---|---|---|---|---|
| M | 16 | 22* | 9.2 | 14.1 |
| M | 17 | 1500 | 4.4 | 12.7 |
| M | 35 | 130 | 12.5 | – |
| M | 35 | 3422 | 5.9 | 12.7 |
| F | | 4210 | 11.3 | – |

**After treatment with pyridoxine, this may have been higher prior to treatment.*

HLA-linked haemochromatosis is a common cause of increased iron absorption from the gut resulting in toxic deposits in tissues. In the North European population this can be diagnosed easily in over 90% of cases by PCR detection of a single mutation (HFE Cys282Tyr).[70] Such a prevalent gene (gene frequency 0.05) will certainly occur amongst patients of the same ethnic origin with XLSA. Detailed studies of the iron status, HFE and ALAS2 mutations in members of a large number of families have not yet been published, however, a preliminary report[71] indicates that the coinheritance of the HFE Cys282Tyr gene is not required for iron loading to occur. That coinheritance of haemochromatosis may enhance the degree of iron loading was suggested by a recent report of iron loading in two brothers with XLSA.[72] One was heterozygous for haemochromatosis and developed a more severe iron overload at an earlier age than the one without. Much larger studies are required to substantiate the contribution of heterozygous HFE mutations to the haemochromatosis of XLSA. Furthermore, the issue is clouded by the existence of HLA-linked haemochromatosis not associated with the North European common mutation.[73]

Iron overload is a common feature of the disorder irrespective of the degree of anaemia and also may affect female heterozygotes who are only slightly anaemic (see, for example Table 3).[56,74-76] Once it occurs treatment can be difficult requiring desferrioxamine in those with severe organ dysfunction and phlebotomy in others. A combination of the two can also be used. For phlebotomy to be effective at removing iron it has to be used at such a rate as to keep ahead of the already increased iron absorption that will increase even more (to 10 mg per day) during the treatment. This requires a

phlebotomy schedule of at least one unit a week for a considerable length of time (depending on the size of the iron stores) for treatment to be effective.[77,78] Once the patient has been made iron deficient then a less strenuous schedule can presumably be employed to keep the patient in iron balance. Unfortunately there are not yet any studies published on effective long-term management of these patients. Desferrioxamine therapy is required in patients with iron-induced organ damage but toxicity at low iron levels prevents this being used to make iron stores normal. To avoid pathological complications it is essential to prevent the iron overload by monitoring iron stores in both the anaemic patients and the female carriers and by removing the excess iron regularly.

There are now several reports of iron overload in pyridoxine-responsive XLSA and other dyserythropoietic anaemias associated with a decrease in effective erythropoiesis which can be relieved by removal of excess body iron.[58,77-82] The mitochondrial iron which stains blue in the Perls stain can be decreased by limiting iron supply to the erythroblast either by chelation or phlebotomy.[77] Maintaining a low transferrin saturation in patients with XLSA will therefore decrease the pool of potentially toxic iron within the mitochondrion. In one iron loaded patient with XLSA a response to pyridoxine was not observed until his serum ferritin was at the low end of normal and in another maximal red cell haemoglobinisation occurred when the transferrin was least saturated.[58] It seems possible therefore that mitochondrial iron in excess of protoporphyrin IX production may be particularly damaging to the ALAS catalytic reaction and such an effect might be magnified in patients whose ALAS2 activity is more severly compromised. Avoidance of iron overload is not only essential to prevent its toxic effect on several organs of the body but it may have an additional bonus for these patients in particular in that it allows erythropoiesis to be optimised by eliminating an iron-mediated inhibition of haem synthesis.

## Diagnosis of XLSA

Mutation detection can be achieved by studying either cDNA derived from the RNA of reticulocytes[55] or genomic DNA.[23]

Reticulocyte ALAS2 mRNA can be reverse transcribed into cDNA and amplified in overlapping sequences using primer sequences that are specific for the erythroid form of ALAS.[55,56] The disadvantage of this is that occasionally in females a mutation may interfere with primer annealing and cause unnoticed loss of amplification of the abnormal allele. Also, mutations within the 5' and 3' flanking regions of the gene, intronic branch point sequences and the sequences flanking the exons containing splice site sequences will be missed. Transport of blood for RNA extraction and the handling of RNA is not as straightforward as for DNA however this is becoming increasingly routine making this less of a problem.

Mutation detection by PCR amplification of genomic DNA and direct cycle sequencing is the best current method for detecting all mutations except for a complete gene deletions in one allele in the heterozygous state. Such cases (none reported to date) would require Southern blotting with genomic probes. Primers for amplifying and sequencing exons 4,[22] 5,[47] 7,[48] and 9,[23] with their flanking sequences, and recently for amplifying all the exons, intron-exon boundaries, promoter and 3' flanking regions have been published.[41] The strategy involves PCR amplification of all 11 ALAS2 exons and the gene's 5' and 3' flanking regions followed by direct cycle sequencing using primers that anneal sufficiently distal in the intron sequences to assure detection of branch site and splice junction mutations. Automated fluorescent DNA sequencing and computer-aided analysis of aligned electropherograms using such programmes as the Applied Biosystems-Perkin Elmer "Autoassembler" or Gene Codes "Sequencer" permit accurate diagnosis in as short as 2 weeks of intensive effort. Cloning of PCR fragments prior to sequencing is not recommended as extra effort is required to sequence multiple clones to rule out errors due to misincorporation with *Taq* polymerase. Sensitive methods for detection of mutations in DNA fragments such as single strand conformation polymorphism (SSCP) analysis can facilitate rapid screening when faced with large numbers of patients and thus direct sequencing efforts to those exons which show a mobility change. However, these methods do not detect 100% of mutations and may not work as well for some regions, depending on their location and base-composition.

Confirmation that any mutation detected is present in that person's DNA and is not an artefact of the amplification, cloning and/or sequencing procedures is best accomplished by PCR of the region and detection of a restriction enzyme site alteration caused by the mutation or introduced by utilization of a mismatched primer.[47,56] In the rare cases where this approach is not possible, allele-specific oligonucleotide hybridization can be used with both wild type and mutant probes.[23]

Once the mutation is confirmed then the same methodology can be used to test other family members rapidly for their carrier status. At least one hundred normal alleles (50 DNA samples from unrelated females) should also be tested to ensure that this is not a common polymorphism. In the absence of showing affected family members with the mutation, some caution is needed in concluding that a mutation is causative of XLSA. More confidence can be gained if the mutated enzyme is

expressed in recombinant systems and demonstrated to have reduced activity or stability.

In the absence of a mutation in the sequences routinely examined, or the lack of available sequencing facilities, polymorphic sites within or close to the ALAS2 gene can be used to try to establish or discount linkage to the gene amongst affected family members. Such sites include a highly informative dinucleotide repeat within intron 7[83] and a polymorphic site 220 nucleotides 3' to the polyadenylation signal detected by *Xmn I* restriction after mismatch oligonucleotide primed amplification.[84]

### *When is molecular diagnosis required?*

Any patient who is suspected as having XLSA should have their DNA or reticulocyte RNA tested for ALAS2 mutations irrespective of sex and age. This includes anyone with a non-haemolytic, microcytic, hypochromic anaemia, or with an anaemia which has a microcytic, hypochromic component, in the presence of normal or raised iron stores, and in the absence of any indication of thalassaemia, haemoglobin instability or chronic disease. DNA-based diagnosis not only gives the patient a firm conclusion ending speculation and unecessary investigation and possible misdiagnosis, but the means are then also provided for rapid testing other family members who may be at risk of iron overload even if they do not have manifest anaemia. Accurate diagnosis and experience with the consequences seen if other families with the same or similar mutations enables the family concerned to be educated about the disorder and its likely consequences for them.

In the case of middle age and late-onset forms of the disorder, a distinction between refractory anaemia with ringed sideroblasts (RARS), an acquired disorder associated with an increased risk of leukaemic transformation, and XLSA, an easily treatable inherited one will be warmly welcomed. Reports exist now of three patients with inherited mutations in the ALAS2 gene who had been wrongly diagnosed as having refractory anaemia with ringed sideroblasts. These are the two late onset patients with complete haematological responses to pyridoxine described above[48] and a female heterozygous for the arginine 411 to cysteine mutation.[66] Factors other than the apparent rarity of this condition compared with RARS which contribute towards misdiagnosis include a lack of appreciation of the variable age of onset of some forms of XLSA, an apparent thrombocytosis due to the recording of a flagged automated platelet count that was including small red cells and an inability to demonstrate the heritability of the phenotypic change in an obligate heterozygote due to the mildness of the defect[48] (Figure 4).

## Family studies and genetic counselling

The analysis of red blood cell morphology cannot be relied upon routinely for the diagnosis of heterozygotes. Skewed Lyonisation may cause a female heterozygote to have as severe a disorder as a male member of the same family if it is the X chromosome that carries the normal ALAS2 gene that is predominantly inactivated. Conversely, it can also cause the sideroblastic cells to be so few as to be



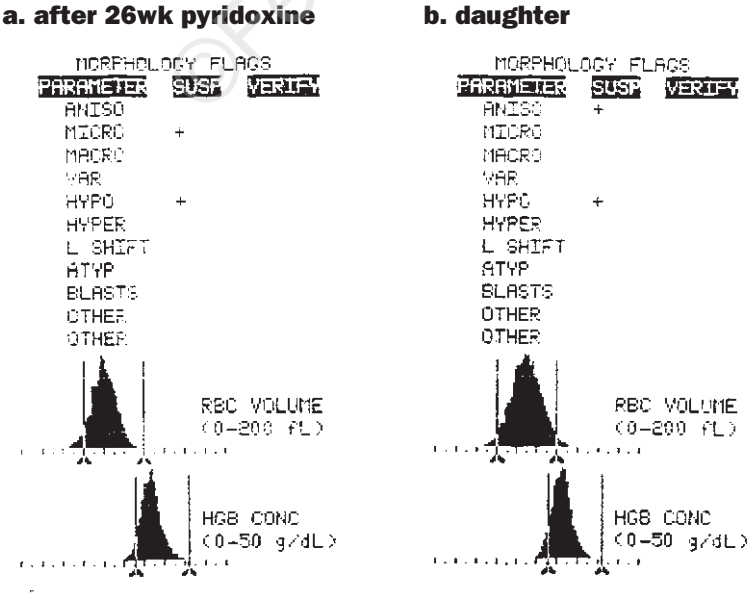


**Figure 4. The red cell size histograms in a patient (a) with a complete haematological response to pyridoxine and (b) his daughter. These were obtained using the Bayer Technicon H3 automatic cell analyser. Although the red cell size distribution was broader than usual in the daughter, encompassing an increased proportion of hypochromic cells, sufficient bimodality (see for comparison Figure 1D) was not present for the staff concerned to be confident that the father had an inherited form of sideroblastic anaemia.[46]**

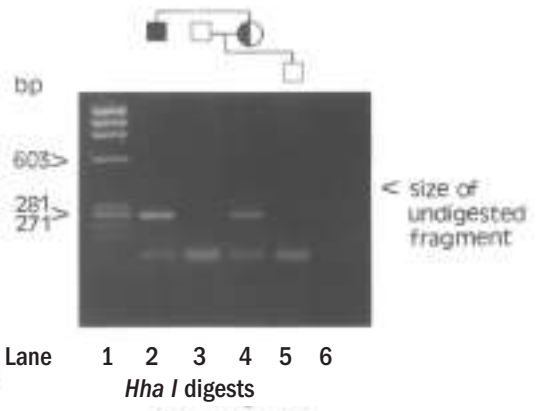


**Figure 5. The use of PCR to confirm the 1283 C→T mutation in exon 9 of the ALAS2 gene predicted to substitute arginine 411 by cysteine and to test for its presence in the newborn son of a carrier female relative. A fragment of 416 base pairs encompassing exon 9 was amplified and digested with the restriction endonuclease *Hha I*. The mutation abolishes one of the two restriction sites normally present in this fragment, leaving a mutation-specific fragment of 261 base pairs present in the original patient (lane 2) and the mother (lane 4) of the child. The newborn child was male but had not inherited the gene for XLSA (lane 5). Lane 1 is a size marker and lane 6 is a no-DNA control.**

undetectable when the X chromosome that is predominantly inactivated is the one carrying the mutant ALAS2. Definitive genetic counselling advice therefore requires that a diagnosis of the carrier state is made at the DNA level. Women generally want to know if their child is likely to be affected but prenatal diagnosis is unlikely to be requested except for the pyridoxine-refractory severe XLSA or by someone from a family affected by a form of pyridoxine-responsive XLSA that has been associated with considerable morbidity and mortality. Were prenatal diagnosis requested however, DNA diagnosis of the mutation or a closely linked marker will be essential.

Neonatal diagnosis of an affected child would enable families and pysicians to prevent unecessary and innappropriate treatment such as iron supplements or blood transfusion, allow early administration of pyridoxine and allow for the regular monitoring of iron loading in order to prevent it by phlebotomy. If the child was not affected then this would save anxiety. Indeed neonatal testing was requested recently by a pregnant woman who was heterozygous for the arginine 411 to cysteine abnormality (Figure 5). The neonate was a boy who was found not to be affected within a few days of receiving the sample. This avoided prolonged uncertainty regarding the expected blood picture at birth for this particular mutation and the need for follow up blood samples.

It should be stressed that the iron accumulation of XLSA is life-threatening if untreated, with onset varying from infancy to over 80 years of age in both affected males and carrier females. The family members should receive counselling to educate them of the risks to both hemizygotes and heterozygotes and should be encouraged to inform other family members at risk about the availability of testing and the need to measure iron stores. The iron overload can be prevented if diagnosis occurs early enough and if it has already occurred it is usually amenable to treatment by chelation therapy and by phlebotomy. Phlebotomy is important especially when iron levels reach the toxicity threshold for the chelator, during the maintenance phase once toxic tissue iron stores have been reduced and as the means of iron loading prevention.

## Conclusions

Pyridoxine-responsive XLSA is a rare condition and as such may be overlooked or not managed optimally. Until recently, the heterogeneity of sideroblastic anemia was confusing but now with pyridoxine-responsive XLSA it is clear that the cause is missense mutations in a gene which has a key regulatory role in erythroid haem biosynthesis. Mutation detection enables the early diagnosis of those affected, targetted education of families, early treatment with pyridoxine and prevention of iron overload. It also allows for a distinction to be made between late-onset variants of this condition and the more insidious refractory anaemia with ring sideroblasts.[88]

The recent findings of ALAS2 mutations in pyridoxine-refractory XLSA which may be severe and found only in carrier females highlights the need to consider this as a cause, albeit a rare one, of unexplained repeated miscarriage or early neonatal death. Sequence analysis has revealed erythroid-specific cis elements in the ALAS2 promoter,[89] an iron regulation system involving an IRE in the 5' untranslated part of the mRNA, and putative haem responsive motifs in the N-terminal leader peptide of the protein. The scene is thus set for potential revelations regarding erythroid commitment, differentiation and intracellular iron-haem-globin homeostasis. It is to be hoped that the causes of the iron overload in these patients may become better understood and prevented more easily. Where population screening programmes for haemoglobinopathies[90,91] and haemochromatosis include careful haematological scrutiny, in particular of the red cell indices, people affected by XLSA will be found and this may provide a fortuitous route to their early diagnosis and iron overload prevention. In all, advances in molecular diagnosis and characterization are paving the way for improved mutation detection, patient management and ultimately, gene therapy.

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*Alison May and David F Bishop equally contributed to this review article.*

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