British Journal of Haematology, 1999, 105, 75–79

A case of complete adenylate kinase deficiency due to a nonsense mutation in *AK-1* gene (Arg 107 → Stop, CGA → TGA) associated with chronic haemolytic anaemia

PAOLA BIANCHI, MANUELA ZAPPA, ELENA BREDI, CRISTINA VERCELLATI, GIOVANNI PELISSERO, FIORENZA BARRACO AND ALBERTO ZANELLA *Divisione di Ematologia, IRCCS Ospedale Maggiore, Milano, Italy*

Received 3 August 1998; accepted for publication 4 January 1999

Summary. Two siblings of Italian origin with mild chronic haemolytic anaemia, psychomotor impairment and undetectable adenylate kinase (AK) activity are reported. The other red cell enzyme activities were normal except for a slight decrease of PFK. 2,3-DPG levels were increased in both siblings, and AMP decreased in one only. The parents were not consanguineous and displayed intermediate AK activity. The sequence of complete erythrocyte *AK-1* cDNA showed the presence of a nonsense homozygous mutation at codon 107 (CGA → TGA, Arg → Stop) in the siblings. The mutation results in a truncated protein of 107 amino acids in comparison with the 194 of the normal one. Moreover a 37 bp deletion in the first part of exon 6 (from nt 326 to nt 362 of the cDNA sequence) was detected in one allele; this deletion is not likely to further affect the enzyme structure, being localized after the stop codon. The new variant was named AK Fidenza, from the origin of the patients.

Keywords: adenylate kinase deficiency, chronic haemolytic anaemia, erythrocyte metabolism, nonsense mutation, *AK-1* gene.

Adenylate kinase (ATP-AMP phosphotransferase, EC 2.7.4.3) (AK) is an ubiquitous enzyme which catalyses the reversible transfer of phosphate moiety among ATP, ADP and AMP and contributes to the homeostasis of the adenine nucleotide composition in the cell. In vertebrates three isoenzymes have been characterized: AK1, expressed in the cytosol of skeletal muscle, brain, and erythrocyte; AK2, in the intermembrane space of mitochondria of liver, kidney spleen and heart, and AK3, present in the mitochondrial matrix of liver and heart (Russel *et al*, 1974).

The human erythrocytic isoenzyme AK1 is encoded by a 12 kb long gene (*AK-1*), which is split into seven exons (Matsuura *et al*, 1989); the *AK-1* gene generates two mRNA species of 0·9 and 2·5 kilobases respectively, which differ at the 3′ portion.

Six AK1-deficient families have been reported (Szeinberg *et al*, 1969; Boivin *et al*, 1971; Kende *et al*, 1982; Beutler *et al*, 1983; Miwa *et al*, 1983; Lachant *et al*, 1991; Toren *et al*, 1994; Qualtieri *et al* 1997), but only in two was the molecular defect identified as missense mutations CGG-TGG Arg128-Trp and TAT-TGT Tyr164-Cys (Matsuura *et al*, 1989; Qualtieri *et al*, 1997). From a clinical point of view, erythrocyte AK deficiency is associated with chronic nonspherocytic haemolytic anaemia in all cases but one (Beutler *et al*, 1983), and with psychomotor impairment in some patients only (Boivin *et al*, 1971; Toren *et al*, 1994). However, the direct relationship between the reduced erythrocyte survival and the enzyme defect has been questioned by some authors and other concomitant causes of haemolysis have been hypothesized (Beutler *et al*, 1983; Lachant *et al*, 1991; Toren *et al*, 1994).

In this paper we describe an Italian family in which two siblings, born of non-consanguineous parents, carried a homozygous nonsense mutation resulting in a truncated protein with complete lack of AK activity, associated with mild congenital nonspherocytic haemolytic anaemia and psychomotor impairment.

MATERIAL AND METHODS

Case report. The propositus was a 7-year-old boy of Northern Italian origin with a history of mild chronic haemolytic anaemia and occasional need for blood transfusions during infectious episodes. At the time of the study the Hb was 10·8 g/dl, reticulocytes 278×10^9/l, unconjugated bilirubin 22·23 μmol/l. Screening for abnormal or

Correspondence: Dr Alberto Zanella, Divisione di Ematologia, Padiglione Granelli, IRCCS Ospedale Maggiore, Via F. Sforza 35, 20122 Milano, Italy. e-mail: div_emat@polic.cilea.it.

Table I. Some clinical and haematological data of the propositus and his family members.

	Proband	Sister	Father	Mother	Reference values
Age (year)	7	8	37	40	
Neonatal jaundice	No	No	No	No	
Splenomegaly	No	No	No	No	
Transfusion (no. of units)	4	0	0	0	
Psychomotor abnormalities	Yes	Yes	No	No	
Haemoglobin (g/dl)	10·8	11·4	17·1	10·8	12·1–16·7
VGM (fl)	90	91	90	66	
Reticulocytes (10^9/l)	278	203	16	64	24-84
Unconjugated bilirubin (μmol/l)	22·23	14·36	8·721	6·84	<12·82
Haptoglobin (μmol/l)	< 3·1	< 3·1	23·5	34·8	9·3–46·5
Lactate dehydrogenase (IU/l)	1150	568	320	360	230–460
Haemoglobin A2 (%)	2·0	2·7	2·8	4·2	1·3–3·7
Erythropoietin (U/l)	19	34	nd	nd	5–30
Ferritin (μg/l)	30	64	133	29	19–255
Autohaemolysis (%)	0·2	1·7	0·8	1·7	2–5
+ glucose	1·5	1·3	0·2	0·7	0·2–2
+ ATP	1	0·5	0·7	0·9	0·2–2
Osmotic fragility	Normal	Normal	Normal	Decreased	
Heat stability	Normal	Normal	Normal	Normal	
Spectrin/band 3	0·95	0·94	0·95	0·95	0·95–1·21
Ankyrin/band 3	0·18	0·23	0·24	0·23	0·13–1·26
Band 4/band 3	0·33	0·31	0·33	0·32	0·28–0·35

unstable haemoglobins, red cell osmotic fragility, autohaemolysis and direct antiglobulin test were negative. The study of the most important red cell enzymes revealed the complete absence of AK activity, the other enzyme activities being normal or elevated in relation to the reticulocytosis. Strictly similar haematological and metabolic findings were detected in his 8-year-old sister. Moreover, psychomotor retardation was detectable in the propositus, and his sister presented with language retardation. The parents were not consanguineous and displayed intermediate AK activity. The mother carried a β-thalassaemic trait. The most important clinical and haematological data at the time of diagnosis are summarized in Table I.

Haematological assays and enzymes studies. Routine haematological investigations were performed according to the method of Dacie & Lewis (1984). The assay of glycolytic and pentose phosphate pathway enzyme activities was performed as reported elsewhere (Zanella *et al*, 1988), following the methods of Beutler (1984), and expressed as IU/10^{10}RBC to partially correct the overestimation in one of the family members (the mother) who carried a β-thalassaemia trait (Vives Corrons *et al*, 1984).

Purification and biochemical characterization of red cell AK and PK were carried out as previously described by Tsuboi & Chervenka (1974) and Miwa *et al* (1979) respectively. Serum iron, total iron binding capacity and transferrin saturation (TS) were determined by standard methods. Serum ferritin was measured by an enzyme immune assay procedure (IMX System, Abbott Laboratories, Abbott Park, Ill.).

The study of red cell membrane proteins was performed as previously described (Bianchi *et al*, 1997). Serum erythropoietin (s-Epo) and transferrin receptor (s-TfR) levels were determined by enzyme-linked immunoassays (s-Epo: Epo-ELISA, Medac Diagnostika, Hamburg, Germany; s-TfR: Clinigen, Amgen Diagnostics, Thousand Oaks, Calif.).

Reference subjects. 50 normal individuals coming from different Italian regions were used as a reference population for the molecular study.

Nucleic acid analysis. Blood samples were collected from the patients, their relatives and a group of normal individuals. Leucocytes were isolated and genomic DNA was extracted using standard manual methods (Sambrook *et al*, 1990).

Total reticulocyte RNA was isolated by ammonium chloride lysis (Boyer *et al*, 1976; Goossens & Kan, 1981) and reverse transcribed using random primers. The complete *AK-1* cDNA was PCR-amplified in two overlapping fragments of 306 bp and 456 bp (Fig 1). Primers used for PCR were: AK1 (sense) CCC AGA GAG CAC TGA CAC GG and AK1B (antisense) CCT TTG GAA GTA TTG ACT TTG GC; AK2 (sense) CTG CCT GAT TAG AGG TCC TCC, and AK2B (antisense) GGA GGA CCT CTA ATC AGG ACG. PCR conditions were: 92°C for 60 s, 58°C for 30 s, 72°C for 40 s, for 30 cycles on a Thermo cycler 480 (Perkin Elmer Cetus, Norwalk, Ct.).

Sequencing reactions were performed using as a template double-strand DNA subcloned with TA Cloning Kit (Invitrogen) on Automated Laser Fluorescence Sequencer using an Autoread sequencing kit (Pharmacia Biotech, Uppsala, Sweden).

The new mutation was confirmed using the restriction enzyme Tsp45I (New England Biolabs, Beverly, Mass.) at the

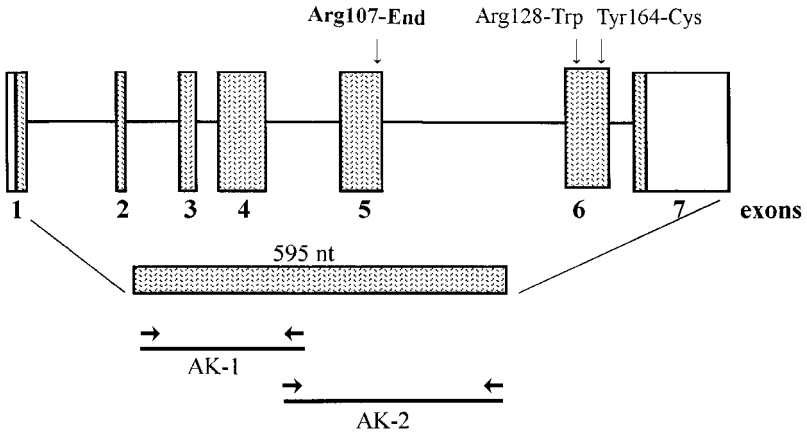

Fig 1. *AK-1* gene structure and position of primers used. Boxes show exons: □, coding regions; ■, non-coding regions. Exons are indicated by numbers. Arrows indicate the position of the mutation described in this study (bold) and that of the other mutations already described in the literature.

genomic level on a PCR-amplified fragment of 200 bp using the following primers: AK5′ ATG CCA TGG TGG CCA AAG TC (sense) and AK3′ TGC TCA GAA CTC TGA CCT GC (antisense). The genomic DNA of 50 normal individuals were investigated for the presence of this mutation.

To investigate the effect at the genomic level of the 37 nt deletion (from nt 10075 to nt 10111 of the genomic sequence), a set of primers (sense) internal to intron 5 were prepared: AK9911: GAG TGG CAG GAA AGA TTG T, AK9773: ACA GCA TGA GCA CAG GTC TG, AK8941: ATC CTT AAC TAC GGA GGA GC, AK7986: GGT GCC ACA GGT CTG AAA GA, AK6860: GCA GGT CAG AGT TCT GAG CA, and primer AK6681 (exon 5): ATG CCA TGG TGG CCA AAG TC; and amplified by PCR with the antisense primer AK10189: TGA TGG TCT CCT CAT TGT CG located in exon 6.

RESULTS

Red cell enzyme and metabolic intermediate assays

The results of enzyme assays performed on the family members are summarized in Table II.

AK activity was nil in the propositus and his sister, and intermediate in the parents. The activity was completely absent also on semipurified AK samples. The other enzymatic activities were normal or slightly increased in relation to reticulocytosis except for a slightly decreased PFK values in the propositus, sister and mother.

The determination of the most important metabolic intermediates showed increased levels of 2,3-DPG in both children, and markedly decreased AMP concentration in the propositus only.

Table II. Red cell enzyme and metabolic intermediate assays in all the family members.

	Proband	Sister	Father	Mother	Reference values*
Red cell enzymes (IU/10^{10} RBC)					
Hexokinase	0·58	0·97	0·40	0·78	0·24–0·39
Glucosephosphate isomerase	20·02	21·42	14·16	14·16	11·51–16·32
Phosphofructokinase	2·34	2·34	3·50	2·24	2·51–3·74
Triosephosphate isomerase	413·22	nd	nd	nd	388·6–566·0
Glyceraldehyde-P-dehydrogenase	78·71	78·99	70·10	76·65	39·15–66·90
Phosphoglycerate kinase	104·76	173·53	94·21	126·14	73·37–97·15
Pyruvate kinase	4·27	5·33	3·62	9·63	3·19–4·52
Glucose-6-phosphate dehydrogenase	2·96	3·72	2·82	3·47	1·98–2·64
6-Phosphogluconic dehydrogenase	3·37	2·54	2·61	2·83	2·13–2·84
Adenylate kinase	0	0	38·49	32·76	51·91–78·01
Enolase	1·51	1·60	1·30	1·29	1·22–1·79
Phosphoglucomutase	0·79	1·01	0·85	0·71	0·59–0·812
Diphosphoglucomutase	1·68	1·82	1·92	1·39	1·34–1·87
Metabolic intermediates					
2,3-Diphosphoglicerate (nmol/10^{10} RBC)	6358	6035	3746	4318	2258–3555
ATP (nmol/10^{10} RBC)	1264	1156	1382	827	1044–1409
ADP (nmol/10^{10} RBC)	163	182	204	191	130–194
AMP (nmol/10^{10} RBC)	12·55	19·90	15·90	30·00	19·1–38·2
Pyruvate (mmol/10^{10} RBC)	26·04	26·90	36·65	14·41	11·48–22·79

* Values expressed as 2·5 and 97·5 centile of the reference distribution.

AK electrophoresis, performed in both parents, did not reveal any abnormal band.

DNA study

The sequence of complete erythrocyte *AK-1* cDNA showed the presence of a nonsense homozygous mutation at codon 107 (CGA-TGA, Arg-Stop) in the propositus and his sister: the same substitution was detectable at the heterozygous level in the parents. The mutation results in a truncated protein of 107 amino acids in comparison with the 194 of the normal one. It creates a site for the restriction enzyme Tsp45 I (Fig 2), and was not detected in the genomic DNA of 50 normal individuals. The new variant was named AK Fidenza, after the town of origin of the patient.

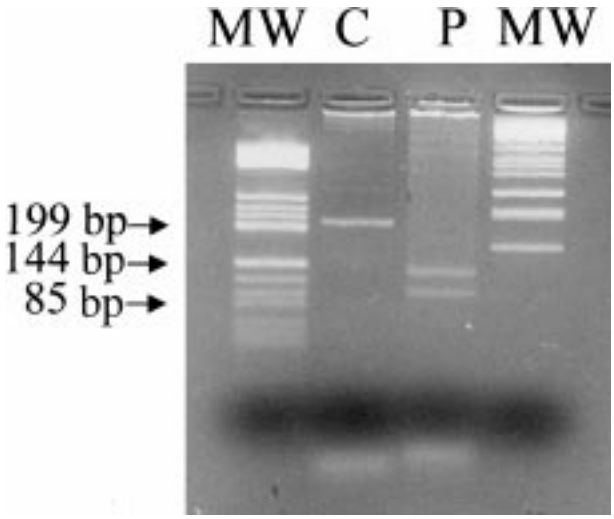

Fig 2. *Tsp45 I* digested PCR fragments obtained from the proband and normal control to confirm the presence of the new mutation. C = normal control, P = patient, MW = molecular weight marker. Normal band = 199 bp; mutated bands: 85 bp and 144 bp.

Moreover, in the cDNA, a 37 bp deletion in the first part of exon 6 (from nt 326 to nt 362 of the cDNA sequence) was detected.

To investigate the origin of this abnormality at the genomic level, a set of internal PCR reactions from intron 5 to exon 6 was performed. In no case was a second band, corresponding to the deleted allele, present. The sequences of the intronic regions flanking exons 5 and 6 were normal.

Moreover a difference at nt 10022 (c–g) in intron 5 from the genomic sequence published by Matsuura *et al* (1989) was found. The nucleotidic substitution creates a restriction site for BseRI enzyme and was detected in all the controls studied.

DISCUSSION

In this paper we describe a new variant of adenylate kinase completely lacking enzymatic activity in two siblings with mild congenital haemolytic anaemia and psychomotor impairment.

Mild to severe haemolytic anaemia occurs in all the AK-deficient patients so far reported, with one exception. In fact, one of the two siblings described by Beutler *et al* (1983) had no signs of haemolysis in spite of the complete absence of enzyme activity. Following this observation, the cause–effect relationship between AK deficiency and hyperhaemolysis has been questioned. Some authors claimed that the shortened survival of AK-deficient RBC was caused, or at least enhanced, by the coexistence of defects of other enzymes such as phosphorybosil pyrophosphate synthetase, pyruvate kinase and AMP:GTP phosphotransferase (Lachant *et al*, 1991), G6PD (Szeinberg *et al*, 1969; Toren *et al*, 1994), or other unidentified factors (Matsuura *et al*, 1989).

In the present case no red cell abnormalities other than AK deficiency were detected, in spite of extensive membrane and metabolic studies. The slightly decreased PFK activity is probably the consequence of the increased 2,3-DPG levels (Mentzner & Glader, 1989) that are almost constantly found in AK deficiency (Beutler *et al*, 1983; Lachant *et al*, 1991). The remote possibility that a PK dysfunction could account for the increased 2,3-DPG was ruled out by the normal results of PK biochemical characterization.

The only two molecular abnormalities so far identified in AK deficiency are missense mutations Arg128-Trp and Tyr164-Cys (Matsuura *et al*, 1989; Qualtieri *et al*, 1997).

Generally speaking, the cause-and-effect relationship between a single amino acid substitution and the enzyme defect is, however, not certain.

The nonsense mutation found in our patients fully explains the biochemical phenotype and definitely shows that AK gene inactivation causes a complete lack of AK activity and, in turn, haemolytic anaemia. In fact, this is the most drastic molecular abnormality of the *AK-1* gene so far reported, and its relationship with the lack of enzyme activity is unequivocal. The homozygous mutation results in a truncated protein of 107 amino acids in comparison with the 194 of the normal one. The gene product is likely to be inactive and not to survive to the intracellular proteolysis, in line with the *in vivo* finding of a complete absence of AK activity. Moreover, the observation that this mutation was not detected in the normal population (100 alleles examined) rules out the possibility of a polymorphism and further supports the correlation between the molecular finding and the enzyme defect.

Unexpectedly, the parents deny consanguinity in spite of the fact that they share the same, rare, mutation. Indeed, an accurate genealogical analysis six generations backward failed to demonstrate any link between lineages. However, since the two families originate from the same small geographic area, consanguinity cannot be excluded.

The cDNA study of *AK-1* gene also revealed the presence of a 37 nt deletion at the beginning of exon 6. The deletion, although it modifies the frame of the protein determining the creation of a stop codon at aminoacid 125, is not likely to further affect the enzyme structure, being localized after the nonsense mutation.

To investigate the origin of this deletion we sequenced the intronic flanking regions of exons 5 and 6 (most commonly involved in splicing), without detecting abnormalities.

Moreover, no large genomic mutation was found in intron 5. Also the possibility of a polymorphism (Fields & Harris, 1966; Bowmann *et al*, 1967), although improbable for the nature of the defect, was excluded by the cDNA study of normal individuals. It could therefore be hypothesized that the nonsense mutation, localized −6 bp at the end of exon 5, determines *per se* the activation of a cryptic splice site. However, we cannot exclude that an additional undetected mutation in the intronic sequences may be responsible for the abnormal splicing.

The AK-deficient siblings here described displayed psychomotor impairment, language retardation and limited learning abilities. Mental retardation from mild to severe was also found in all the six homozygous AK deficient siblings studied by Toren *et al* (1994). Moreover, mental impairment was present in the case described by Boivin *et al* (1971), although it was attributed to the forceps delivery and resuscitation at birth. Since AK1 isoenzyme is expressed in red cells and brain, we can suppose that the mutation found in our patients could have caused abnormalities in normal brain development or function.

ACKNOWLEDGMENT

This work was supported by research grant R.C.95 160/01 from IRCCS Ospedale Maggiore of Milan.

REFERENCES

Beutler, E. (1984) *Red Cell Metabolism: a Manual of Biochemical Methods*. Grune & Stratton, New York.

Beutler, E., Carson, D., Dannawi, H., Forman, L., Kuhl, W., West, C. & Westwood, B. (1983) Metabolic compensation for profound erythrocyte adenylate kinase deficiency: a hereditary enzyme defect without hemolytic anemia. *Journal of Clinical Investigation*, 72, 648–655.

Bianchi, P., Zanella, A., Alloisio, N., Barosi, G., Bredi, E., Pelissero, G., Zappa, M., Vercellati, C., Baronciani, L., Delaunay, J. & Sirchia, G. (1997) A variant of the EPB3 gene of the anti-Lepore type in hereditary spherocytosis. *British Journal of Haematology*, 98, 283–288.

Boivin, P., Galand, C., Hakim, J., Simony, D. & Selingman, M. (1971) Un nouvelle erythroenzymopathie anémie hémolitique congenitale non spherocitaire et deficit héreditaire. *La Presse Medicale*, 79, 215–218.

Boyer, S.H., Noyes, A.N. & Boyer, M.L. (1976) Enrichment of erythrocytes of fetal origin, from adult–fetal blood mixtures via selective hemolysis of adult blood cells: an aid to antenatal diagnosis of hemoglobinopathies. *Blood*, 47, 883–897.

Bowman, J.E., Frisher, H., Franco, A., Carson, P.E. & Gower, M.K. (1967) Population, family and biochemical investigation of human adenylate kinase polymorphism. *Nature*, 214, 1156–1158.

Dacie, J.V. & Lewis, S.M. (1984) *Practical Haematology*. Churchill Livingstone, London.

Fields, R.A. & Harris, H. (1966) Genetically determined variation of adenylate kinase in man. *Nature*, 208, 261–263.

Goossens, M. & Kan, Y.Y. (1981) DNA analysis in the diagnosis of hemoglobin disorders. *Methods in Enzymology*, 76, 805–817.

Kende, G., Ben-Bassat, I., Brok-Simoni, F., Holtzman, F. & Ramot, B. (1982) Adenylate kinase deficiency associated with non-spherocytic haemolytic anaemia. *Proceedings of the XIXth International Meeting of the Society for Haematology*, 19, 224.

Lachant, N., Zerez, C.R., Barredo, J., Lee, D.W., Savely, S.M. & Tanaka, K.R. (1991) Hereditary erythrocyte adenylate kinase deficiency: a defect of multiple phophotransferase? *Blood*, 77, 2774–2784.

Matsuura, S., Igarashi, M., Tanizawa, Y., Yamada, M., Kishi, F., Kajii, H., Miwa, S., Sakurai, M. & Nakazawa, A. (1989) Human adenylate kinase deficiency associated with hemolytic anemia: a single base substitution affecting solubility and catalytic activity of the cytosolic adenylate kinase. *Journal of Biological Chemistry*, 264, 10148–10155.

Mentzner, W.C. & Glader, B.E. (1989) Disorders of erythrocyte metabolism. In: *The Hereditary Haemolytic Anaemias* (ed. by W. C. Mentzner and G. M. Wagner). Churchill Livingstone, London.

Miwa, S., Boivin, P., Blume, K.G., Arnold, H., Black, J.A., Kahn, A., Staal, G.E., Nakashima, K., Tanaka, K.R., Paglia, D.E., Valentine, W.N., Yoshida, A. & Beutler, E. (1979) Recommended methods for the characterization of red cell pyruvate kinase variants. *British Journal of Haematology*, 43, 275–279.

Miwa, S., Fujii, H., Tani, K., Takahashi, K., Takizawa, T. & Igarashi, T. (1983) Red cell adenylate kinase deficiency associated with hereditary nonspherocytic hemolytic anemia: clinical and biochemical studies. *American Journal of Hematology*, 14, 325–333.

Qualtieri, A., Pedace, V., Bisconte, M.G., Bria, M., Gulino, B., Andreoli, V. & Brancati, C. (1997) Severe erythrocyte adenylate kinase deficiency due to homozygous A–G substitution at codon 164 of human AK-1 gene associated with chronic haemolytic anaemia. *British Journal of Haematology*, 99, 770–776.

Russel, P.Y., Jr, Horenstein, J.M., Goins, L., Jones, D. & Laver, M. (1974) Adenylate kinase in human tissue. *Journal of Biological Chemistry*, 249, 1874–1879.

Sambrook, J.T., Fritsch, E.F. & Maniatis, T. (1990) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

Szeinberg, A., Kahana, D., Gavendo, S., Zaidmann, J. & Ben Ezzer, J. (1969) Hereditary deficiency of adenylate kinase in red blood cells. *Acta Haematologica (Basel)*, 42, 111–126.

Toren, A., Brok-Simoni, F., Ben-Bassat, I., Holtzman, F., Mandel, M., Newmann, Y., Ramot, B., Rechavi, G. & Kende, G. (1994) Congenital haemolytic anaemia associated with adenylate kinase deficiency. *British Journal of Haematology*, 87, 376–380.

Tsuboi, K.K. & Chervenka, C.H. (1974) Adenylate kinase of human erythrocyte. *Journal of Biological Chemistry*, 250, 132–140.

Vives Corrons, J.L., Pujades, M.A., Aguilar, I., Bascompte, J.L., Jou, J.M., Rozman, C. & Ester, A. (1984) Pyrimidine 5′-nucleotidase and several other red cell enzyme activities in α-thalassaemia trait. *British Journal of Haematology*, 56, 483–494.

Zanella, A., Colombo, M.B., Miniero, R., Perroni, L., Meloni, T. & Sirchia, G. (1988) Erythrocyte pyruvate kinase deficiency: 11 new cases. *British Journal of Haematology*, 69, 399–404.