bjh review

Hereditary pyrimidine 5′-nucleotidase deficiency: from genetics to clinical manifestations

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Summary

Hereditary pyrimidine 5′-nucleotidase (P5′N) deficiency is the most frequent abnormality of the red cell nucleotide metabolism causing hereditary non-spherocytic haemolytic anaemia. The disorder is usually characterised by mild-to-moderate haemolytic anaemia associated with the accumulation of high concentrations of pyrimidine nucleotides within the erythrocyte. The precise mechanisms leading to the destruction of P5′N deficient red cells are still unclear. The pyrimidine 5′-nucleotidase type-I (P5′N-1) gene is localised on 7p15-p14 and the cDNA has been cloned and sequenced; 20 mutations have been identified so far in 30 unrelated families, most of them at the homozygous level. Recently, the comparison of recombinant mutants of human P5′N-1 with the wild-type enzyme has enabled the effects of amino acid replacements on the enzyme molecular properties to be determined and help to correlate genotype to clinical phenotype.

Keywords: pyrimidine 5′-nucleotidase, mutations, erythroenzymopathy, hereditary haemolytic anaemia.

Inherited P5′N-1 deficiency, transmitted as an autosomal recessive trait, is the most frequent enzyme abnormality of the red cell nucleotide metabolism, and is thought to be the most common cause of chronic non-spherocytic haemolytic anaemia after glucose 6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) deficiency (Vives Corrons, 2000). Since the first description (Valentine *et al*, 1974), more than 60 patients with P5′N-1 deficiency have been reported worldwide (Vives Corrons, 2000; Marinaki *et al*, 2001; Balta *et al*, 2003; Bianchi *et al*, 2003; Rees *et al*, 2003; Kanno *et al*, 2004; Manco *et al*, in press), with presumably large numbers undetected (Marinaki *et al*, 2001). Clinical symptoms occur only in homozygous and compound heterozygous patients. The prevalence of P5′N-1 deficiency is not known.

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Structure and function of P5′N-1

The 5′-nucleotidases (5′-NT, nucleoside 5′-monophosphate phosphohydrolase, EC 3·1·3·5) are catabolic enzymes that catalyse the dephosphorylation of non-cyclic nucleoside 5′-monophosphates to their respective nucleosides and inorganic phosphate, thus regulating cellular nucleotide and nucleoside levels. To date, at least seven different 5′-NT with distinctive substrate specificity, distribution, cellular localisation, regulation and amino acid sequences have been identified in mammals. One of them is a membrane-bound form, located on the cell surface via a glycosylphosphatidylinositol anchor (Misumi *et al*, 1990; Zimmermann, 1992), whereas the other six are intracellular. Among intracellular 5′-NT, five are cytosolic (Rampazzo *et al*, 2000a; Hunsucker *et al*, 2001; Sala-Newby & Newby, 2001; Amici *et al*, 2002; Bretonnet *et al*, 2005) and one occurs in mitochondria (Rampazzo *et al*, 2000b). Moreover, some 5′-NT are ubiquitous; others are expressed in a tissue-specific manner (Bianchi & Spychala, 2003; Hunsucker *et al*, 2005). Most 5′-NT exhibit quite broad substrate specificity and require magnesium for activity (Hunsucker *et al*, 2005). Furthermore, some 5′-NT display the activity of transferring phosphate groups from nucleotide donors to nucleoside acceptors (Amici *et al*, 1997; Bretonnet *et al*, 2005). Analysis of the 5′-NT amino acid sequence demonstrated that all intracellular 5′-NT are characterised by the presence of a DXDX(T/V) motif, a distinctive signature of a number of other phosphatases/phosphotranspherases (Collet *et al*, 1998; Rinaldo-Matthis *et al*, 2002). This conserved motif is thought to be the enzyme catalytic site in which the first aspartate residue is transiently phosphorylated upon catalysis (Collet *et al*, 1998; Allegrini *et al*, 2001; Amici *et al*, 2005; Bretonnet *et al*, 2005). All 5′-NT characterised so far, except P5′N-1, exhibit oligomeric structure with identical subunits of about 20–60 kDa. A summary of the advances on the structure, cellular function, and specific properties of individual 5′-NT has been recently reported (Bianchi & Spychala, 2003; Hunsucker *et al*, 2005).

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Two main types of 5′-NT, both active towards pyrimidine nucleotides, have been isolated from red blood cells (Paglia *et al*, 1984; Hirono *et al*, 1985, 1987; Amici *et al*, 1997) (Fig 1). These two enzymes, often considered in the older literature as a single entity, are coded by two distinct genes (Wilson *et al*, 1986; Marinaki *et al*, 2001) and display different specificity with respect to the sugar present in pyrimidine nucleotides. P5′N-1 is mainly active towards pyrimidine 5′-ribonucleotides (Amici *et al*, 1997) whereas P5′N type-2 (P5′N-2), also known as 5′(3′)-deoxyribonucleotidase, is not strictly pyrimidine-specific and is more efficient with deoxyribonucleotides (Rampazzo *et al*, 2000a).

Both enzymes are ubiquitous, although P5′N-1 is often thought an erythrocyte-specific enzyme, being mainly studied in erythrocytes (Hunsucker *et al*, 2005).

To date, the haemolytic anaemia caused by P5′N deficiency has been related only to a defective P5′N-1 activity in red blood cells.

The major role of P5′N-1 is found in the catabolism of the pyrimidine nucleotides, mainly resulting from ribosomal RNA degradation during final erythroid maturation (Paglia & Valentine, 1975; Mass *et al*, 2003; Rees *et al*, 2003). The pyrimidine nucleotides are not used by mature erythrocytes and need to be transported out of the cells to prevent their accumulation. Differently from pyrimidine nucleotides, the nucleosides can diffuse out of the cells. This fact indicates that P5′N-1 depletes red cells from the excess of nucleotides through a dephosphorylation process (Paglia & Valentine, 1975; Rees *et al*, 2003).

Pyrimidine 5′-nucleotidase type-I activity is much higher in reticulocytes than in old red cells, because of the unique decay pattern during the erythrocyte life span. In fact, contrary to what is observed in the other age-dependent red cells enzymes, the rapid loss of P5′N-1 activity during the first few days of maturation is followed by a further continuous decline throughout the ageing of the cell, as demonstrated by Beutler and Hartman (1985) in transient erythroblastopenia of childhood.

Pyrimidine 5′-nucleotidase type-I, also called cytosolic 5′-nucleotidase III (cNIII) (Bianchi & Spychala, 2003) or uridine monophosphate hydrolase type-1 (UMPH-1) (Swallow *et al*, 1983), has been recently purified to homogeneity as recombinant form. A thorough characterisation of the biochemical properties of the enzyme is now available (Amici *et al*, 2005; Chiarelli *et al*, 2005). It is a protein containing 286 amino acids (TrEMBL accession number Q9P0P5) with no disulphide bridges and no phosphate groups (Amici *et al*, 1997, 2002). P5′N-1 preferentially catalyses the dephosphorylation of uridine monophosphate (UMP) (k_{cat} and K_m, 11·98/s, 0·056 mmol/l respectively) and cytidine monophosphate (CMP) (k_{cat} and K_m, 26·14/s and 0·18 mmol/l respectively) and is totally inactive towards purine nucleotides, regardless of the position of the phosphate moiety (Valentine *et al*, 1974). Interestingly, P5′N-1 also displays phosphotransferase activity

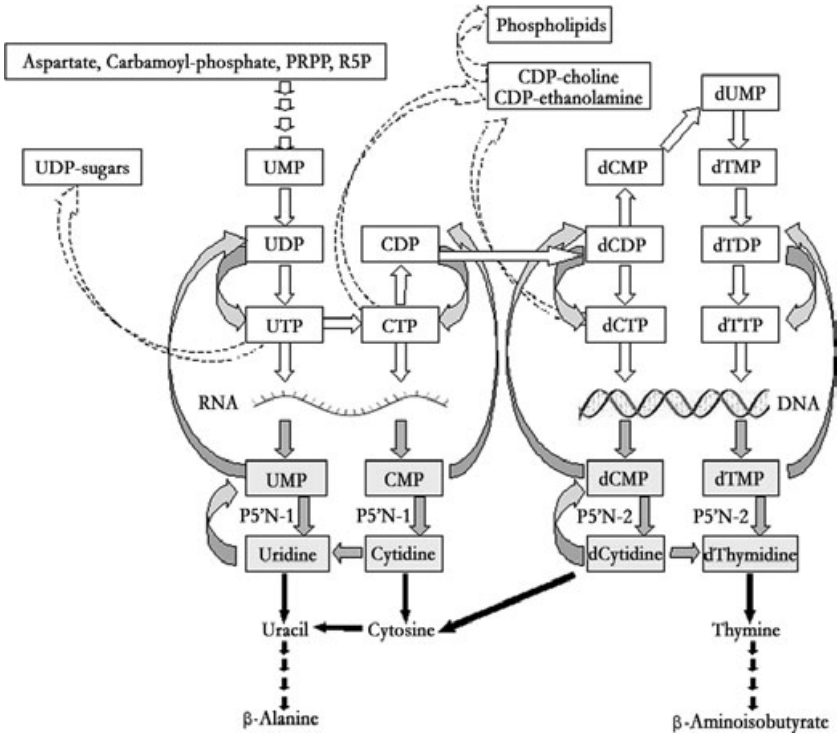

Fig 1. Scheme of pyrimidine metabolic pathways. CTP, cytidine triphosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; TTP, thymidine triphosphate; TDP, thymidine diphosphate; TMP, thymidine monophosphate; PRPP, phosphoribosyl-pyrophosphate; R5P, ribose 5-phosphate; d, deoxy-. White, grey and black arrows indicate *de novo* synthesis, salvage pathway and degradation in the liver respectively.

specific for pyrimidine nucleosides, suggesting an additional role in nucleotide metabolism (Amici *et al*, 1997). This activity, also present in cytosolic 5′-nucleotidase II (Bretonnet *et al*, 2005), but absent in P5′N-2 (Rampazzo *et al*, 2000a), is of clinical interest as pyrimidine nucleoside analogues, such as 3′-azido-2′-deoxythymidine and cytosine arabinoside, are widely used in chemotherapy.

The enzyme is absolutely dependent on magnesium ions (Mg^{2+}) for activity; thus metal chelating reagents, like EDTA, are strongly inhibitory. Mg^{2+} cannot be replaced by Mn^{2+}. The enzyme activity is also readily inhibited by heavy metal ions, mostly Pb^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+} and Cd^{2+}, and by thiol-reactive reagents, such as *N*-ethylmaleimide, *p*-chloromercuribenzoate and 5,5′-dithiobis (2-nitrobenzoic acid) (Valentine *et al*, 1974; Paglia & Valentine, 1975; Torrance *et al*, 1977a; Amici *et al*, 2002). In humans, severe lead poisoning results in P5′N-1 deficiency, which closely resembles the syndrome associated with hereditary deficiency state (Vives Corrons, 2000; Rees *et al*, 2003).

Pyrimidine 5′-nucleotidase type-I displays optimal activity at pH 7·5–8·0, and is most stable between pH 6 and 7·5 (Valentine *et al*, 1974; Paglia & Valentine, 1975; Torrance *et al*, 1977a; Amici *et al*, 2002). Differently from what is previously reported, P5′N-1 is a rather stable enzyme, retaining full activity after 1 h of incubation at 46°C, as well as over a month storage at 4°C. Nevertheless, it is worth noting that the enzyme becomes less stable if the temperature is lowered to −20°C (activity reduced to 60%) (L.R. Chiarelli, unpublished observations). Unfortunately, the crystal structure of P5′N-1 is not yet available, but far-UV circular dichroism analysis shed light on the secondary structure elements, showing that the enzyme consists of approximately 37% alpha-helices and 26% extended beta-strands (Chiarelli *et al*, 2005). Unlike other 5′NT, P5′N-1 exhibits monomeric structure, with an apparent molecular mass of 34 kDa.

Genetic characteristics of pyrimidine P5′N deficiency

The gene encoding P5′N-1 (*NT5C3, UMPH-1*) is located on chromosome 7p15-p14 (Marinaki *et al*, 2001) and spans approximately 50 kb. The codifying region is split into 11 exons (1, 2, R, 3–10) leading to the production of three mRNA forms by alternative splicing of exons 2 and R (Marinaki *et al*, 2001; Kanno *et al*, 2004).

The transcript lacking both exons 2 and R is expressed ubiquitously (Lu *et al*, 2000), whereas transcripts including exon 2 alone or both exons 2 and R are almost specifically expressed in reticulocytes (Kanno *et al*, 2004). The biological significance of these various mRNAs found in reticulocytes is at present unknown; among the three expected proteins (285, 286 and 297 amino acids long) only the protein consisting of 286 amino acid residues, identical to the previously identified lupus inclusion protein p36 (Amici *et al*, 1994, 2000), has been isolated from erythrocytes. It is produced using the initiation codon at the 3′-end of exon 1 and sequentially lengthened from exon 3, both exons 2 and R being spliced out (Marinaki *et al*, 2001; Kanno *et al*, 2004).

It should be noted that the amino acid numbering adopted in this review refers to the spliced form of gene for P5′N-1 consisting of 286 residues (Swiss-Prot accession no. Q9H0P0-2; the numbering of nucleotides is referred to cDNA reported in EMBL (coding sequence): accession no. AAF36153).

To date, 20 different mutations in the P5′N-1 gene have been identified in 37 patients from 30 families affected by P5′N deficiency (Table I). Six of them are missense mutations (c.260A → T, c.392T → C, c.469G → C, c.536A → G, c.688G → C and c.740T → C) and do not affect the protein length; two lead to in-frame amino acid deletion (c.159–167del and c.427–429del), the remaining 12 consist of nonsense mutations, deletions, insertions or alterations of the splicing sites, and are expected to generate truncated or aberrant forms of the polypeptide. All the patients but two are homozygous.

Some mutations have been found to be distributed on a regional basis; in particular, c.543delG was found in the homozygous state in three patients originating from a well-defined geographical area of southern Italy (Bianchi *et al*, 2003), and missense mutation c.688G → C in five families apparently unrelated but all originating from Kyushu island (Kanno *et al*, 2004). Mutation c.710-711insGG has been reported both in Italy and Turkey (Balta *et al*, 2003; Bianchi *et al*, 2003); moreover, in the same nucleotidic position, the insertion of an *Alu* repetitive element of 330 bp has been described in two unrelated Portuguese patients (Manco *et al*, in press). It can be assumed that mutation c.710-711insGG originated from the subsequent excision of the *Alu* sequence.

A codon 81 TAC-TAT polymorphism was found to be linked to a P5′N-1 mutation in a Norwegian family, and a population survey showed that the TAT allele had a frequency of 0·29 in the UK (Marinaki *et al*, 2001).

Pathophysiology

The key metabolic abnormality in P5′N-1 deficiency is the accumulation of pyrimidine nucleotides that precipitate, resulting in massive red cell basophilic stippling. However, the precise mechanism(s) leading to the red cells destruction are still unclear (Vives Corrons, 2000; Rees *et al*, 2003).

It is known that the accumulation of pyrimidine nucleotides has many effects on red cell metabolism (Fig 2). First, it enhances the activity of pyrimidine nucleoside monophosphate kinase, which accounts for the disproportionate increase of the nucleoside triphosphates cytidine triphosphate (CTP) and uridine triphosphate compared with CMP observed in P5′N deficient red cells (Lachant *et al*, 1987). Moreover, the accumulation of CTP results in a marked increase of cytidine diphosphate (CDP)-choline and CDP-ethanolamine, which are important constituents of the red cell membrane. The abnormal membrane phospholipid composition has been suggested as a possible cause of haemolysis (Swanson *et al*,

Table I. Mutations reported in pyrimidine 5′-nucleotidase type 1 gene associated with chronic haemolytic anaemia.

cDNA nucleotide substitution (Marinaki *et al*, 2001)	cDNA nucleotide substitution (EMBL AAF36153)	Effect	Exon(s)	Country	Reference
c.251-252ins A	c.218-219ins A	Frameshift	5	Japan	Kanno *et al* (2004)
c.192-200del	c.159-167del	3 aa del	5	Japan	Kanno *et al* (2004)
c.293A → T	c.260A → T	Asp 87Val	6	Norway	Marinaki *et al* (2001)
c.339G → C	c.306G → C	Trp102Cys, splice site	6	Japan	Kanno *et al* (2004)
c.384-385ins A	c.351-352ins A	Frameshift	7	Turkey	Balta *et al* (2003)
c.425T → C	c.392T → C	Leu131Pro	7	Japan Portugal	Kanno *et al* (2004) and Manco *et al* (in press)
c.429-430del AG	c. 396-397del AG	Splice site	8	Italy	Unpublished observation
c.460-462del CAA	c.427-429del CAA	Gln143 del	8	Italy	Unpublished observation
c.502G → C	c.469G → C	Gly157Arg	8	Portugal	Manco *et al* (in press)
c.529C → T	c.496C → T	Gln166 STOP	8	South Africa	Marinaki *et al* (2001)
c.543T → G	c.510 T → G	Tyr170 STOP	8	Turkey	Balta *et al* (2003)
c.569A → G	c.536A → G	Asn179Ser	8	Italy	Bianchi *et al* (2003)
c.576del G	c.543del G	Frameshift	8	Italy	Bianchi *et al* (2003)
IVS8+1-2del GT	IVS8+1-2del GT	Splice site	IVS8	Bangladesh	Escuredo *et al* (2004)
c.721G → C	c.688G → C	Gly230Arg	9	Japan	Kanno *et al* (2004)
c.743-744ins GG	c.710-711ins GG	Frameshift	9	Italy Turkey	Bianchi *et al* (2003) Balta *et al*, (2003)
c.743-744ins 330bp	c.710-711ins 330bp	ex 9 del	9	Portugal	Manco *et al* (in press)
c.773T → C	c.740T → C	Ile247Thr	9	Portugal	Manco *et al* (in press)
IVS9−1G → C	IVS9−1G → C	Splice site	IVS9	Italy	Bianchi *et al* (2003)
IVS10+1G → T	IVS10+1G → T	Splice site	IVS10	South Africa	Marinaki *et al* (2001)

Nucleotide substitutions are numbered according to Marinaki *et al* (2001) and EMBL accession number AAF36153.

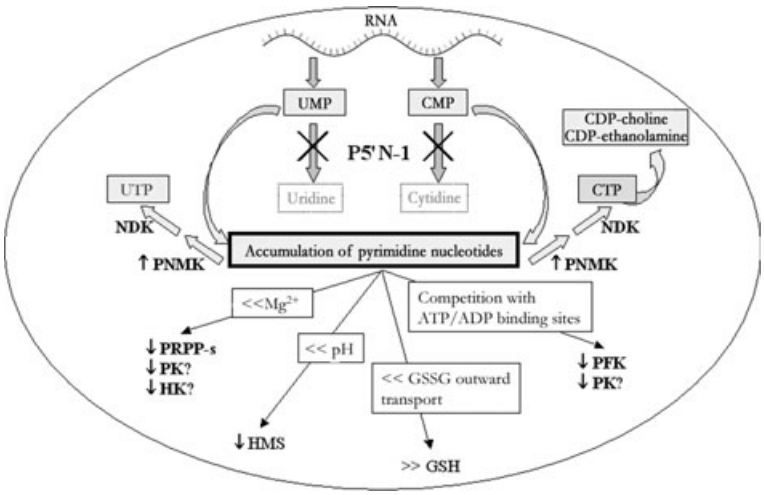

Fig 2. Red cell metabolic abnormalities consequent to pyrimidine 5′-nucleotidase deficiency. CTP, cytidine triphosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; UTP, uridine triphosphate; UMP, uridine monophosphate; PNMK, pyrimidine nucleoside monophosphate kinase; NDK, nucleoside diphosphate kinase; PRPP-s, phosphoribosyl-pyrophosphate synthetase; PK, pyruvate kinase; HK, hexokinase; PFK, phosphofructokinase; GSSG, oxidised glutathione; GSH, reduced glutathione; HMS, hexose monophosphate shunt.

1984), although red cell osmotic fragility is usually not increased in P5′N-deficient patients (Vives Corrons, 2000), with few exceptions (Valentine *et al*, 1973; Bianchi *et al*, 2003).

Pyrimidine nucleotides are also known to be strong chelators of Mg^{2+} and may therefore affect the activity of enzymes requiring Mg^{2+} as a co-factor or Mg-ATP as a substrate. In particular, magnesium sequestration by pyrimidine nucleotides is, at least in part, responsible for the reduced activity of phosphoribosyl-pyrophosphate (PRPP) synthetase found in P5′N-deficient erythrocytes (Lachant *et al*, 1989), which may in turn result in an impairment of the PRPP-

dependent adenine salvage pathway (Zerez *et al*, 1986). CTP has also been shown to inhibit *in vitro* other Mg-dependent enzymes, such as PK and hexokinase (Lachant & Tanaka, 1986), although the activity of these enzymes appears to be normal in P5′N-deficient red cells (Beutler *et al*, 1980; Dvilansky *et al*, 1984; David *et al*, 1991). It has also been suggested that the accumulation of pyrimidine nucleotides may interfere with the glycolytic flux by competing with ATP/ADP-binding sites of the glycolytic enzymes, such as phosphofructokinase and PK (Oda *et al*, 1984), but no conclusive evidence for a metabolic block was found.

An impaired pentose phosphate shunt activity has also been demonstrated in some patients and related to a decreased intraerythrocytic pH due to the accumulated nucleotides. In particular, G6PD activity is lower in P5′N-deficient red cells (Tomoda *et al*, 1982; David *et al*, 1991), especially in young erythrocytes (Tomoda *et al*, 1982; David *et al*, 1991). Whether the impairment of the pentose phosphate pathway may contribute to the pathogenesis of haemolysis of P5′N deficiency is not clear. Rees *et al* (1996) hypothesised that the inhibition of the pentose-phosphate shunt activity in young erythrocytes contributed to the severity of haemolysis in a case of combined HbE and P5′N deficiency.

Finally, increased levels of reduced glutathione have been observed in P5′N deficiency (Valentine *et al*, 1972; Miwa *et al*, 1977) and related to the inhibition of outward transport of oxidised glutathione by pyrimidine nucleotides (Kondo *et al*, 1980, 1987).

Clinical, haematological and diagnostic aspects of P5′N deficiency

Clinical features

Figure 3 summarises the main clinical features of P5′N deficiency, as assessed by the review of the clinical data of 64 patients from 54 families of various origin reported in the literature. The median age at the time of diagnosis was 15 years (range, 3 months to 64 years).

The clinical manifestations comprise the usual hallmarks of lifelong chronic haemolysis. The anaemia was usually mild to moderate, or even fully compensated in rare cases (Kanno *et al*, 2004). Severe anaemia occurred in 12% of patients. The degree of anaemia is relatively constant during the patient's life, although occasional exacerbation may occur during acute infections and pregnancy and may require blood transfusions (Vives Corrons *et al*, 1976; Rechavi *et al*, 1989). Pregnancy is usually well tolerated and associated with favourable perinatal outcome, although transfusions may be required in some cases (Valentine *et al*, 1972; Paglia *et al*, 1980; Ghosh *et al*, 1991).

Jaundice and splenomegaly are common in P5′N deficiency. Half of the patients were splenectomised, most of them before diagnosis, and one-third developed cholelithiasis.

Overall, 17 patients needed blood transfusions, and three became transfusion dependent (David *et al*, 1991; Ghosh *et al*, 1991; Rees *et al*, 1996). Exchange transfusion was required in one case only.

Iron overload can be a predictable complication of chronic transfusion therapy, but it may also occur in patients with limited or no history of transfusions (Bianchi *et al*, 2003), similarly to what is described in other non-spherocytic haemolytic anaemias (Zanella *et al*, 1993, 2001). Iron deficiency secondary to intravascular haemolysis was reported in one case (Ericson *et al*, 1983).

Delayed development and learning difficulties of variable degree were reported in seven patients, which was not related to the severity of haemolysis (Torrance *et al*, 1977b; Beutler *et al*, 1980; Li *et al*, 1991; Balta *et al*, 2003). Database searches have shown the presence of P5′N-1 cDNA libraries in brain tissue (Marinaki *et al*, 2001); however, the significance of the association between mental retardation and P5′N deficiency is hard to assess; possible confounding factors include kernicterus or concomitance of other genetic defects in consanguineous families.

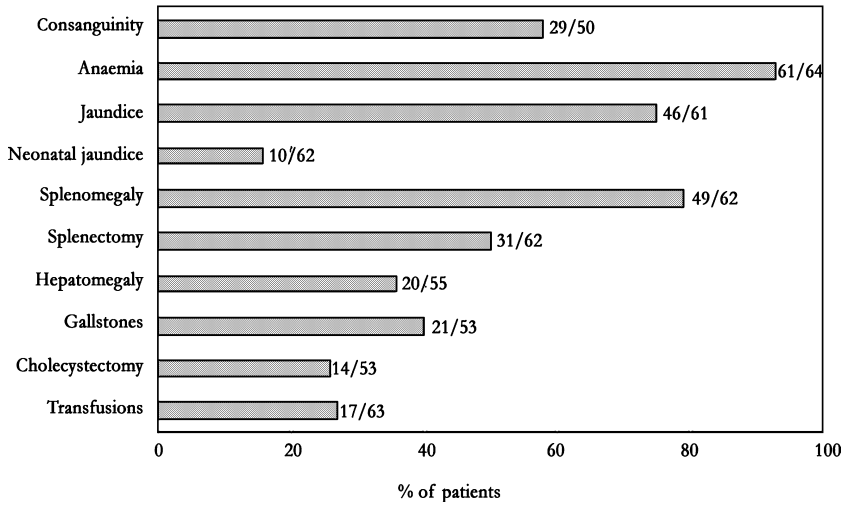

Fig 3. Main clinical data of 64 pyrimidine 5′-nucleotidase-deficient patients reported in the literature.

Aplastic crisis following parvovirus B19 infection (Rechavi *et al*, 1989) and chronic ankle ulcers (Beutler *et al*, 1980) were rare.

Pyrimidine 5′-nucleotidase deficiency has been found in association with HbE (Rees *et al*, 1996), HbD Punjab (unpublished observations) and spectrin deficiency (Bianchi *et al*, 2003). Only in the former case the associated defect greatly exacerbated the clinical pattern.

Haematological features

The haematological features of P5′N deficiency are common to other hereditary non-spherocytic haemolytic diseases, including anaemia, reticulocytosis and biochemical signs of haemolysis. Figure 4 reports some routine laboratory findings of P5′N-deficient patients, divided into splenectomised and unsplenectomised groups.

The median haemoglobin concentration was 9·5 g/dl (range 2·8–15·2 g/dl) without significant differences between splenectomised and unsplenectomised patients. In seven patients examined before and after splenectomy, surgery resulted in stabilisation of the haemoglobin to higher levels (median Hb increase 3·2 g/dl, range 0·5–5·2).

The reticulocyte count is constantly increased and does not appear to be related to the haemoglobin levels (data not shown). Overall, the reticulocytosis tends to be higher in splenectomised than in unsplenectomised patients (median 15% vs. 8%): in most patients examined before and after splenectomy, surgery results in a further increase of reticulocytes. Similar findings have been reported in PK deficiency and referred to a selective sequestration of defective reticulocytes by the spleen (Zanella *et al*, 2005).

Unconjugated hyperbilirubinaemia is higher in splenectomised than unsplenectomised patients (median 72 μmol/l vs. 47 μmol/l) and was reported to increase after splenectomy in two cases (Buc *et al*, 1979; unpublished observations).

The blood film is mainly characterised by red cell basophilic stippling (2–12% of erythrocytes) (Ben-Bassat *et al*, 1976; Marinaki *et al*, 2001; Bianchi *et al*, 2003) due to intracellular precipitation of undegraded RNA (Ericson *et al*, 1983); 5–10% of spherocytes, mostly spiculated, were detected at peripheral blood smear examination in two cases described by Valentine *et al* (1974) and Beutler *et al* (1980) and in the six patients reported by Bianchi *et al* (2003). Red cell osmotic fragility is usually normal with few exceptions (Buc *et al*, 1979; Paglia *et al*, 1980; Bianchi *et al*, 2003).

Iron status parameters have been investigated in eight untransfused P5′N-deficient patients. Three of them, all splenectomised, displayed overt iron overload as assessed by increased iron status parameters (serum ferritin 1620–2400 μg/l; transferrin saturation 61–100%), marked siderosis at liver biopsy and, in one case only, cardiac iron burden at magnetic resonance imaging (Bianchi *et al*, 2003; unpublished observations). Two of them carried *HFE* gene mutations (C282Y/C282Y; H63D/wt). All three patients needed iron chelation.

Diagnostic aspects

The clinical and haematological features of P5′N deficiency are not distinctive, although the blood film gives a strong clue as to the diagnosis when marked red cell basophilic stippling is seen. Basophilic stippling is a constant but not specific finding in this disease, occasionally occurring in other congenital or acquired conditions, such as β-thalassaemia trait (Vives Corrons *et al*, 1984; Joishy *et al*, 1986), some haemoglobin variants (Sozen *et al*, 2004), sideroblastic anaemia or lead poisoning. The diagnosis ultimately depends upon the demon-

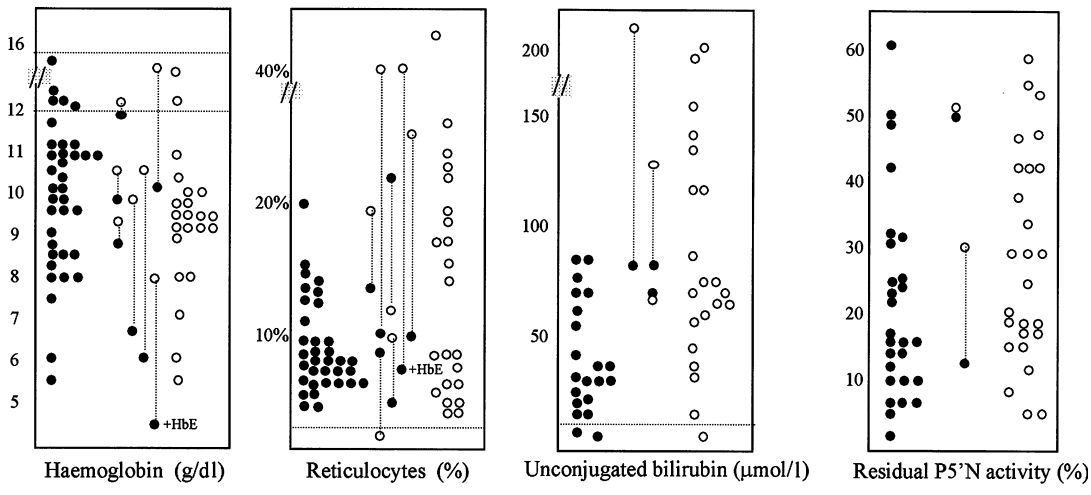

Fig 4. Routine haematological data and pyrimidine 5′-nucleotidase (P5′N) activity of P5′N-deficient patients reported in the literature (●, unsplenectomised; ○, splenectomised). The horizontal dotted lines delimit the reference range for each parameter. Vertical bars indicate pre- and postsplenectomy values in single subject.

stration of high concentrations of pyrimidine nucleotides and a reduced P5′N-1 activity in red blood cells.

The nucleotides of normal red cells consist largely of purine derivatives (which have an absorption maximum at approximately 260 nm), with very low levels of pyrimidine nucleotides (absorption at 280 nm). In P5′N-1 deficiency, high levels of pyrimidine nucleotides accumulate in erythrocytes, resulting in a decrease in the OD260:OD280 adsorbance ratio (Beutler, 1984).

A lower than normal OD260:OD280 ratio was found in all the 28 cases investigated. This test is considered to be specific for P5′N deficiency, and has been recommended by International Committee for Standardisation in Haematology (ICSH) for screening purposes (ICSH, 1989).

There are many different procedures for measuring P5′N-1 activity. They include spectrophotometric determination of the phosphate produced in dephosphorylating reactions (Fiske & Subbarow, 1925; Ames, 1966; Paglia & Valentine, 1975; Zerez & Tanaka, 1985), measurement of radioactive nucleoside liberated from a labelled substrate (Torrance *et al*, 1977c; Buc & Kaplan, 1978; Ellims *et al*, 1978), quantification at 254 nm of nucleosides separated by high-performance liquid chromatography (Simmonds *et al*, 1991; Amici *et al*, 1994), or by means of micellar electrokinetic chromatography (Zanone *et al*, 2004). In particular, the last technique seems to meet the standards of performance and reproducibility that are expected of a clinical test.

Residual P5′N-1 activity has been reported to vary from 1% to 64% (Fig 3) and is not correlated with the degree of the haemolysis (data not shown). The high residual activity observed in some patients could be explained by compensatory mechanisms, such as upregulation of other nucleotidases and/or alternative pathways for nucleotide metabolism (Chiarelli *et al*, 2005). Moreover, P5′N-1 residual activity was not directly correlated with the reticulocyte number (data not shown), supporting the hypothesis that pyrimidine accumulation and metabolic impairment principally occur in the younger erythrocytes of P5′N-deficient patients (David *et al*, 1991).

Finally is noteworthy that, in patients with transient erythroblastopenia of childhood, an incorrect diagnosis of hereditary P5′N deficiency may be made, due to the very low enzyme activity of the older red cell population (Beutler & Hartman, 1985)

Relationship between molecular defect and disease severity

The molecular bases of P5′N-1 deficiency have been recently investigated by studying the biochemical properties of four pathological variants (Asp87Val, Leu131Pro, Asn179Ser and Gly230Arg), obtained by site-directed mutagenesis technology (Kanno *et al*, 2004; Amici *et al*, 2005; Chiarelli *et al*, 2005). All investigated mutant proteins display a reduction of catalytic efficiency, and/or alterations of heat stability. The effects of mutations on the thermal stability of the enzyme and the steady-state kinetics as a function of CMP and UMP are reported in Fig 5, whereas the main parameters produced are summarised in Table II.

Asp87Val enzyme exhibits reduced heat stability and catalytic efficiency especially versus UMP (about 25 times), owing to the increased K_m values. Thus, the replacement of a charged (Asp) with a hydrophobic (Val) amino acid alters both protein stability and kinetic behaviour, accounting for P5′N-1 deficiency. Moreover, as Asp 87 is a well-conserved amino acid, it is conceivable that the mutation c.260A $\rightarrow$ T affects a residue involved in the substrate-binding site.

Leu131Pro protein shows a drastic reduction in thermal stability ($t_{1/2}$ at 46°C about 1 min compared with a fully stable wild type) and kinetic properties slightly altered (k_{cat} values nearly halved and K_m three to five times higher) (Table II). Moreover, this mutant was found to be highly unstable even at 37°C, losing 50% of its activity in about 5 min (Chiarelli *et al*, 2005). Protein stability is particularly important in erythrocytes that lack the ability to synthesise proteins. However, the replacement Lys131Pro is likely to make the enzyme unable to maintain a proper folding, increasing the susceptibility to degradation by ubiquitin–proteasome pathway (Kanno *et al*,

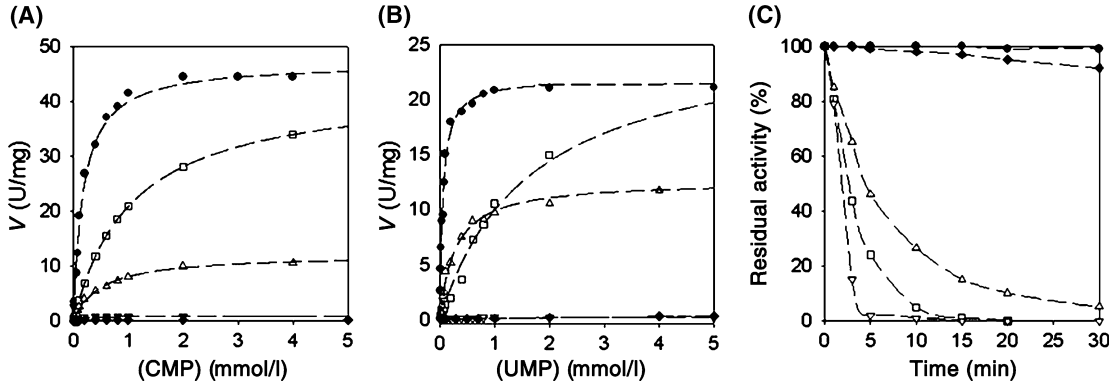

Fig 5. Characterization of wild-type (●) pyrimidine 5′-nucleotidase and four types of mutants (□, Asp87Val; △, Leu131Pro; ■, Asn179Ser; ▽, Gly230Arg). (A) Steady state kinetics as a function of CMP; (B) steady state kinetics as a function of UMP; (C) thermal stability (residual activity after incubation at 46°C expressed as percentage of the initial activity).

Table II. Kinetic and thermal stability parameters of wild type and pyrimidine 5′-nucleotidase type 1 mutants.

	CMP		UMP			
	$k_{cat}(S^{-1})$	K_m(mmol/l)	$k_{cat}(S^{-1})$	K_m(mmol/l)	$t_{1/2}$ (37°C)	$t_{1/2}$ (46°C)
Wild type	26·14 ± 0·70	0·18 ± 0·02	11·98 ± 0·30	0·056 ± 0·006	Stable*	Stable*
Asp87Val	23·30 ± 1·20	1·00 ± 0·10	14·27 ± 0·40	1·640 ± 0·100	25′00″	3′30″
Leu131Pro	6·39 ± 0·20	0·50 ± 0·07	6·86 ± 0·10	0·270 ± 0·020	5′30″	1′20″
Asn179Ser	0·93 ± 0·03	2·60 ± 0·20	0·27 ± 0·01	4·620 ± 0·500	Stable*	Stable*
Gly230Arg	0·24 ± 0·03	0·11 ± 0·02	0·06 ± 0·01	0·100 ± 0·010	Stable*	4′20″

*Maintains more than 90% of activity after 2 h of incubation.
CMP, cytidine monophosphate; UMP, uridine monophosphate.

2004). Therefore, P5′N-1 deficiency associated with mutation c.392T → C appears to be primarily the result of a lowered intracellular level of P5′N-1, rather than the result of the altered kinetic properties of the enzyme.

Asn179Ser enzyme displays an extreme reduction of the catalytic efficiency (more than two to three orders of magnitude), but no alteration in thermostability. As Asn belongs to a region highly conserved among species (Marinaki *et al*, 2001; Mass *et al*, 2003), it is likely that P5′N-1 deficiency is related to an inactive enzyme in which a functionally crucial residue, directly involved in catalysis and substrate binding, has been replaced.

Gly230Arg shows a drastic decay of activity (k_{cat} lowered to <1%), and a significant reduction of thermostability, but no substantial alterations of K_m values. Gly230 is conserved in all known P5′N-1 enzymes, and position 230 probably cannot tolerate the large and charged arginine side-chain introduced by the c.688G → C mutation. Therefore, this unfavourable amino acid replacement accounts for the full inactivation of the enzyme and the reduction of its intracellular level, providing a reason for P5′N-1 deficiency.

In conclusion, based on these findings, all mutations investigated are expected to lead to a decay of intracellular P5′N-1 activity. Therefore, this is the case for Asp87Val and Asn179Ser, with patients exhibiting a very low nucleotidase residual activity (2–5%, Table II). In contrast, the other two replacements, Leu131Pro and Gly230Arg, seem to allow red cells to maintain a rather high activity (up to about 60%), despite the substantial changes in the kinetic and thermostability parameters of mutant enzymes. In the absence of other evidence, this suggests that in some cases P5′N-1 deficiency could be compensated, possibly by other nucleotidases or by alternative pathways in nucleotide metabolism. Therefore, the nucleotidase activity may not be considered a prognostic indicator in P5′N-deficient patients.

Treatment

No specific therapy for P5′N deficiency is available, and the treatment of this disease is therefore based on supportive measures; transfusion dependence is very rare, although occasional transfusions may be required when the anaemia is exacerbated by intercurrent infections, pregnancy or other conditions. Splenectomy does not arrest haemolysis and is generally reported to scarcely improve clinical conditions with a few exceptions (McMahon *et al*, 1981; Ozsoylu & Gurgey, 1981; Dvilansky *et al*, 1984; Rees *et al*, 1996); in particular, two of three regularly transfused patients became transfusion independent after surgery.

Pyrimidine 5′-nucleotidase-deficient patients should be monitored with regard to the iron status, particularly after splenectomy, and screened for the HFE genotype to identify subjects with potential additional risk factors for iron accumulation; repeated courses of iron chelation therapy may be required in these subjects.

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