bjh review

G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications

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Summary

That primaquine and other drugs can trigger acute haemolytic anaemia in subjects who have an inherited mutation of the glucose 6-phosphate dehydrogenase (G6PD) gene has been known for over half a century: however, these events still occur, because when giving the drug either the G6PD status of a person is not known, or the risk of this potentially life-threatening complication is under-estimated. Here we review briefly the genetic basis of G6PD deficiency, and then the pathophysiology and the clinical features of drug-induced haemolysis; we also update the list of potentially haemolytic drugs (which includes rasburicase). It is now clear that it is not good practice to give one of these drugs before testing a person for his/her G6PD status, especially in populations in whom G6PD deficiency is common. We discuss therefore how G6PD testing can be done reconciling safety with cost; this is once again becoming of public health importance, as more countries are moving along the pathway of malaria elimination, that might require mass administration of primaquine. Finally, we sketch the triangular relationship between malaria, antimalarials such as primaquine, and G6PD deficiency: which is to some extent protective against malaria, but also a genetically determined hazard when taking primaquine.

Keywords: G6PD, pharmacogenetics, Clinical Implications.

Over one century ago, Paul Ehrlich found that methylene blue was an effective anti-malarial (Guttmann & Ehrlich, 1891); and in the 1920s, the synthesis of 8-aminoquinolines, specifically plasmoquine and primaquine, was a major advance in the management of malaria (Vale *et al*, 2009). The first large-scale use of primaquine (PQ) took place in the 1950s when US troops were deployed in areas of Korea where malaria was endemic (Jones *et al*, 1953): today it would be called mass drug administration (MDA). Army doctors observed that, apart from minor gastrointestinal complaints, the drug was generally well tolerated; but some of the soldiers receiving the drug became jaundiced and anaemic. This was relatively more common among African Americans, and this complication, toxic effect or side effect, was called the *primaquine sensitivity* syndrome (Dern *et al*, 1955). A.S. Alving's group (Chicago) performed experiments in human volunteers in order to detail the clinical and haematological features (see Fig 1) of the acute haemolytic anaemia (AHA) triggered by PQ (Tarlov *et al*, 1962) (the volunteers were inmates of a penitentiary near Chicago who were clinically and haematologically normal at the start of the experiment, but suffered AHA as a result of the experiment. The volunteers were under constant medical supervision; as far as is known they did not come to any other harm, they all recovered without any blood transfusion and, like after an attack of favism, there is no reason to presume that they had any long-term ill effects. One of us has known in person some of the physicians involved, and regards them as of high moral standards. Nevertheless, this was human experimentation without any medical benefit for the volunteers, and would almost certainly not be allowed today). The same group also reported that the enzymatic activity of glucose 6-phosphate dehydrogenase (G6PD) was markedly reduced in red cells from PQ sensitive subjects (Carson *et al*, 1956); hence the term *G6PD deficiency*. Genetic analysis demonstrated that G6PD deficiency was inherited as an X-linked trait (Adam, 1961).

Pharmacogenetics deals with genetically determined variation in how individuals respond to drugs, in terms of both therapeutic effects and adverse effects. This concept had emerged when tasters and non-tasters of phenylthiocarbamide (PTC) had been identified, and the ability to feel that taste was shown to be inherited (Blakeslee, 1932). As testing for PTC tasting was easy and minimally invasive, PTC tasting was one of the first traits widely studied at the dawn of human population genetics. But it was with the discovery of G6PD deficiency as the biochemical basis of PQ sensitivity that this became a prototype study case in pharmacogenetics,

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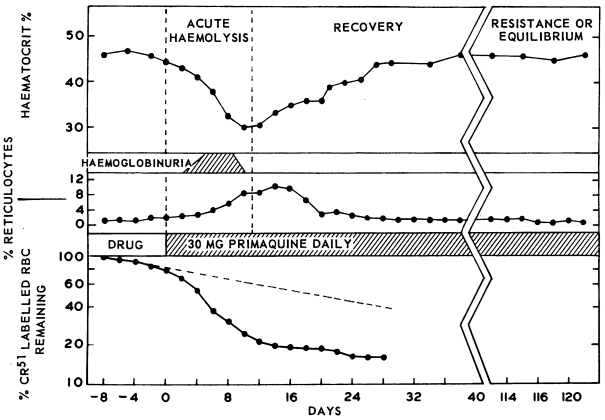

Fig. 1. Clinical course of acute haemolytic anaemia in an adult volunteer receiving primaquine. Reproduced from Tarlov *et al* (1962) Primaquine sensitivity. Glucose-6-phosphate dehydrogenase deficiency: An inborn error of metabolism of medical and biological significance. With permission from the JAMA Network.

just at about the time this term was coined (Motulsky, 1957; Vogel, 1959). Almost immediately it became clear that G6PD deficiency was also the biochemical defect underlying favism (Sansone & Segni, 1958), an acute syndrome associated with anaemia, haemoglobinuria and jaundice, potentially fatal especially in children, intriguingly affecting only some of the many people who eat fava beans. Favism had been known for centuries (Fermi & Martinetti, 1905), but it had been mis-interpreted for a long time as an allergic reaction.

In the 1960s and 1970s, numerous drugs other than PQ were reported as possible triggers of AHA in G6PD-deficient subjects. When a new drug is introduced, 'adverse events' or side effects can be expected. The medicine package sheet is not very helpful, because its compilation is largely driven by medico-legal motives: thus, relatively trivial common complaints (e.g.'headache', 'abdominal discomfort') are listed along with very rare serious problems (e.g. 'acute depression and suicide'). On the other hand, journal publications required for licensing must include tables with the frequency of each side effect as observed in formal trials. The power of pharmacogenetics is that sometimes it can assign a specific meaning to these frequency figures. Thus, the frequency of AHA in patients receiving dapsone may be 10%; but if we stratify the patients by G6PD status, and we find that those 10% were all G6PD deficient, it means that the risk of AHA is practically 0 in G6PD normal patients, and about 100% in G6PD-deficient patients – the original figure of 10% was simply the frequency of G6PD deficiency in the patient population where the trial was conducted. This obvious interpretation of '% risk' must be always before us. Favism, neonatal jaundice and other manifestations of G6PD deficiency are outside the scope of this review.

Drug-induced AHA

Clinical course

The main features of drug-induced AHA are well known (Luzzatto & Poggi, 2009). The two drugs for which we have extensive detailed data are PQ and dapsone. As mentioned above, the clinical course of PQ-induced AHA (Fig 1) has been studied in a number of African-American normal volunteers (Tarlov *et al*, 1962). The clinical picture was very similar to that of favism, which was already well known (Luisada, 1941; Meloni *et al*, 1983): except that with favism the severity is extremely variable, certainly at least in part because the amount of fava beans consumed is highly variable; with PQ 45 mg/day the pattern was instead fairly uniform (Tarlov *et al*, 1962). All subjects had a drop in Hb of about 50 g/l, with a nadir on day 7; they all had haemoglobinuria for 1–3 d; they all developed characteristic changes on the blood film [see Fig 2; and (Bain, 2010)]; they all became jaundiced; they all had a brisk reticulocyte response.

With dapsone the clinical-haematological picture in a similar group of African-American volunteers was very similar (Degowin *et al*, 1966). In addition, there has been recent experience with dapsone-induced AHA (see Fig 3) in a much larger and very different group of G6PD-deficient subjects (Pamba *et al*, 2012). These were children rather than adults; they were not normal volunteers, but patients with acute *Plasmodium falciparum* malaria recruited into multi-centric randomized controlled trials comparing different antimalarial regimes conducted in a number of African countries (Alloueche *et al*, 2004) (Premji *et al*, 2009) (Tiono *et al*, 2009) with a combination of chlorproguanil and dapsone, or with a combination of chlorproguanil, dapsone and artesunate (there is clear evidence that the cause of haemolysis was the dapsone within these combinations). In terms of the quantitative parameters of AHA mentioned above (drop in Hb, haemoglobinuria, bilirubin, reticulocytes) the changes observed were remarkably similar in these children compared to adult volunteers. What was strikingly different was the clinical picture: partly because they already had acute *P. falciparum* malaria, and partly because they developed drug-induced AHA on top of the anaemia (secondary to malaria and to other causes) that most of them already had before starting treatment. As a result, many of the children became severely anaemic and increasingly prostrated, and about 10% of the G6PD-deficient hemizygous males had to be rescued by blood transfusion. The chlorproguanil-dapsone combination that had been licensed in 2003 in 17 African countries under the name of Lapdap was taken off the market in 2008 [see (Luzzatto, 2010)].

With both PQ and dapsone the severity of AHA is markedly dose-dependent (Beutler, 1959; Degowin *et al*, 1966).

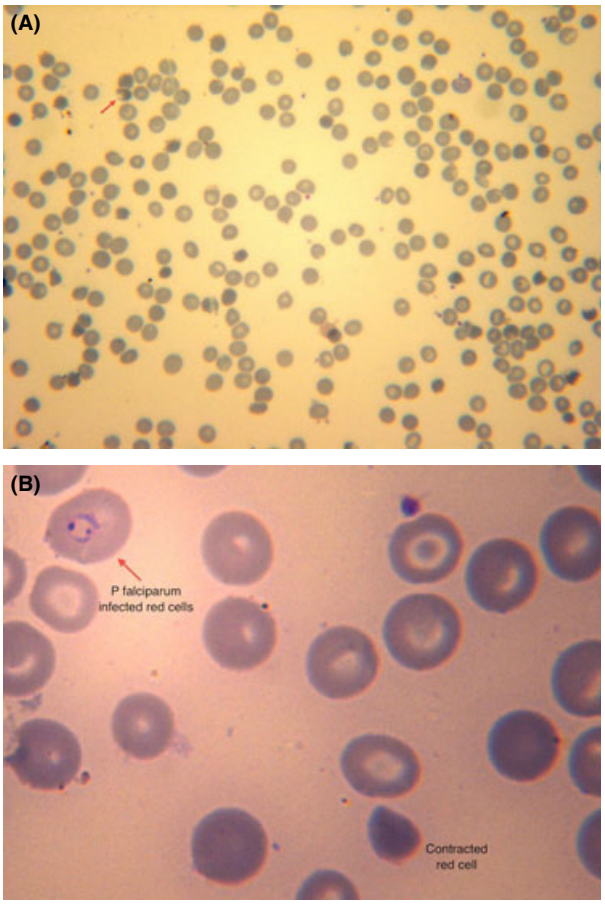

Fig. 2. Blood film from a 3-year-old G6PD-deficient boy with acute uncomplicated *Plasmodium falciparum* malaria. (A) On day 3 after treatment with a chlorproguanil-dapsone combination (see Pamba *et al*, 2012), numerous spherocytes, contracted red cells and haemighosts (arrow) are seen. (B) On day 1, at higher magnification, one sees a *P. falciparum* ring-parasitized red cell and a severely contracted red cell. Blood films courtesy of Dr A B Tiono, Centre National de Recherche et de Formation sur le Paludisme, Ouagadougou, Burkina Faso.

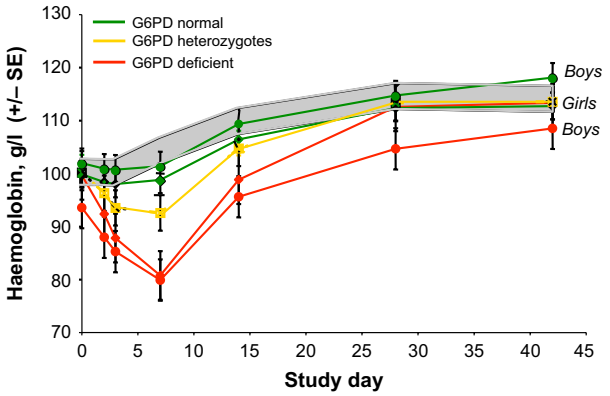

Fig. 3. Clinical course of acute haemolytic anaemia in children with malaria receiving an antimalarial containing dapsone (2·5 mg/kg/day for 3 d). There were 95 G6PD-deficient hemizygous boys, 24 G6PD-deficient hemizygous girls and 200 girls heterozygous for G6PD deficiency. The extent of the drop in haematocrit value in the first two groups indicates that, on average, about 25% of red cells have undergone haemolysis. Thirteen children required blood transfusion. Note that by the end of 6 weeks the blood counts are back to normal, with Hb values higher than before treatment, presumably as a result of the successful treatment of malaria. The shaded area shows the range of Hb over time observed in a group of children with malaria treated with an antimalarial not containing dapsone. Modified from research originally published in Pamba *et al* (2012). © the American Society of Hematology.

Pathophysiology

Given that, in the steady state, the blood of a G6PD-deficient person is normal, drug-induced AHA is the paradigm of a pharmacogenetic event: haemolysis results from the action of an exogenous factor on latently but intrinsically abnormal red cells. The role of G6PD in red cells is to provide reductive potential in the form of NADPH (Berg *et al*, 2002): in G6PD-deficient red cells the NADPH supply is just adequate in the steady state, but it falls short upon exposure to PQ. We can infer that PQ (or another drug) poses an oxidative challenge that G6PD normal red cells can withstand, but G6PD-deficient red cells cannot (see Figs 2 and 4). PQ does not cause haemolysis *in vitro*, and therefore the stepwise sequence of events from oxidative attack to haemolysis have not been fully documented. However, we know that the first crucial biochemical step in a haemolytic episode is decrease of NADPH (Gaetani *et al*, 1979) and the consequent depletion of glutathione (GSH) through its conversion to glutathione disulfide (GSSG) (Beutler, 1957). Once GSH is exhausted, the sulfhydryl groups of haemoglobin and probably of other proteins are oxidized to disulfides or sulfoxides, resulting in coarse precipitates of denatured haemoglobin, Heinz bodies (see Fig 2B) and the formation of haemichromes, which cause irreversible damage to the membrane: specifically, abnormal proteolytic activity is associated with increased intracellular calcium, as well as binding of haemichromes to band 3 molecules. The final clinically important outcome is destruction of red cells, which is both intravascular (witness haemoglobinuria) and extravascular [witness hyperbilirubinaemia (Fischer *et al*, 1985)]. Probably the most severely damaged red cells haemolyse in the bloodstream on their own, whereas less severely damaged red cells will be recognized as abnormal by macrophages and will be their prey in the reticulo-endothelial system.

Several factors can influence the severity of AHA

Drug dose, G6PD activity and red cell ageing. As already mentioned, the dose of a drug is a major determinant of the severity of AHA, because the intensity of the oxidative attack determines how many red cells will be destroyed (Kellermeyer *et al*, 1962). In addition, the schedule according to which the total dose is delivered is also important, and this is mainly related to the fact that red cell destruction in AHA associated with G6PD deficiency is an orderly function of red cell age: the oldest red cells with the least G6PD are the

first to haemolyse, and the haemolytic process progresses upstream toward cells with more and more G6PD activity. Thus, with a single dose of 45 mg PQ there may be abrupt haemolysis of, say, 45% of all red cells in a particular G6PD-deficient person; if instead we give that same person 15 mg PQ, only some 15% of red cells will haemolyse. As PQ is short-lived, if we now give a further dose of 15 mg, many of the surviving red cells will not be sensitive to the corresponding blood level of PQ: therefore there will be less haemolysis, and this will be even more true with the next dose, because with each dose there is a selective enrichment in red cells that, despite being genetically G6PD deficient, have relatively higher levels of G6PD. This phenomenon can be so marked with certain G6PD variants that patients in the post-haemolytic state are found to be relatively resistant to further challenge (Tarlov *et al*, 1962); thus, the patient may be in a state of compensated haemolysis, also called *resistance phase.*

Heterozygous versus *hemizygous (or homozygous) G6PD deficiency.* As the *G6PD* gene is on the X chromosome, males have only one allele, and if this has a mutation causing G6PD deficiency all the red cells will be G6PD deficient. In areas where G6PD deficiency is common, there is a non-negligible number of homozygous females (Luzzatto & Allan, 1968), in whom both *G6PD* alleles are mutant: again all their red cells will be G6PD deficient. (For brevity in this review when discussing hemizygous males we will imply that the same applies to homozygous females). Heterozygous females, instead, by virtue of X chromosome inactivation in somatic cells, have a dual population, with G6PD normal and G6PD-deficient red cells in their blood (Beutler *et al*, 1962): the average ratio is 50:50 but it is well known that the ratio is very variable (Nance, 1964; Rinaldi *et al*, 1976). One can expect therefore that, with a given dose of a given drug, AHA in heterozygotes will be still manifest but will be, on the average, less severe. At one extreme, in a female with a large excess of G6PD normal red cells, haemolysis may be all but undetectable; at the other end of the spectrum, with a large excess of G6PD-deficient red cells, AHA may be just as severe as in hemizygous G6PD-deficient males. These expectations have been verified in a recent study of 200 heterozygotes exposed to dapsone (Pamba *et al*, 2012).

Genetic (allelic) heterogeneity of G6PD deficiency. There are now 187 known mutations in the *G6PD* gene (Minucci *et al*, 2012a), and at least 35 of the mutant alleles are polymorphic, i.e. relatively common in different parts of the world. None of the mutations in these alleles causes complete loss of G6PD, which would be lethal (Pandolfi *et al*, 1995); rather, they cause marked deficiency of G6PD in red cells by decreasing the stability of the variant enzyme (Luzzatto *et al*, 2001). In other words, the normal process whereby G6PD activity decreases as red cells age (see above) is greatly accelerated (Morelli *et al*, 1978). Thus, if old red cells have much less G6PD activity than reticulocytes in G6PD normal blood, the oldest red cells in G6PD-deficient blood will have nearly zero activity. In addition, some mutations significantly affect the binding of substrates (glucose 6-phosphate and NADP), or catalytic efficiency of the enzyme, or both (Mason *et al*, 2007). Indeed, each mutant allele produces a G6PD variant protein that, compared to normal G6PD, has a distinct pattern of quantitative and qualitative changes. In view of this, it would not be surprising if a G6PD-deficient subject with one G6PD variant responded to a drug somewhat differently from another G6PD-deficient subject who has a different G6PD variant. The only available documented comparative data are on the effect of PQ on (i) subjects with G6PD A-, the variant common in Africa, present in the African-American volunteers in Chicago (see above); and (ii) subjects from Sardinia with G6PD Mediterranean, a variant common in that area, in the Middle East and in India. For the same dose of PQ, by comparison to previous experience with G6PD A-, AHA was considerably more severe with G6PD Mediterranean (Pannacciulli *et al*, 1965); in addition, with the latter there was little evidence of a 'resistance phase' upon repeated administration (Salvidio *et al*, 1972). This clinical difference is consistent with the fact that the residual G6PD activity in red cells is about 5% of normal with G6PD Mediterranean, whereas it is 13% of normal with G6PD A- (Luzzatto *et al*, 2001). In view of the fact that G6PD variants differ from each other not only in quantity but also in quality (see above), we cannot assume that the severity of AHA will be a simple function of residual activity. However, because most of the polymorphic G6PD variants for which quantitative data are available have less residual activity than G6PD A-, we must expect in first approximation that, other things being equal, AHA with other variants may be more severe than with G6PD A-.

It must be noted that when discussing genotype-phenotype correlations with respect to G6PD-related drug-induced AHA there is sometimes confusion between two separate issues. The term genotype might refer to hemizygotes and homozygotes *versus* heterozygotes (item 2 above), whereby the difference in clinical phenotype is drastic. By comparison, when the term genotype refers to one *G6PD* allele *versus* others (item 3 above) the differences are rather marginal, especially as it is now clear that, even with G6PD A-, hitherto often regarded as a 'mild' variant, the clinical manifestation can be anything but mild (Pamba *et al*, 2012).

Current list of drugs that can cause AHA

The list of drugs that can cause AHA in G6PD-deficient subjects (Table I) is similar to, but shorter than, many that have been published since the initial World Health Organization (WHO) report (Betke *et al*, 1967). Over the years some drugs have been taken off the list simply because they are no longer in use, and others, especially some antibacterials, because AHA that had been attributed to them was more

Table I. Drugs that can trigger haemolysis in G6PD-deficient subjects

Category of drug	Predictable haemolysis	Possible haemolysis
Antimalarials	Dapsone	Chloroquine
	Primaquine	Quinine
	Methylene blue	
Analgesics/Antipyretic	Phenazopyridine	Aspirin (high doses)
		Paracetamol (Acetaminophen)
Antibacterials	Cotrimoxazole	Sulfasalazine
	Sulfadiazine	
	Quinolones (including nalidixic acid, ciprofloxacin, ofloxacin)	
	Nitrofurantoin	
Other	Rasburicase	Chloramphenicol
	Toluidine blue	Isoniazid
		Ascorbic acid
		Glibenclamide
		Vitamin K
		Isosorbide
		Dinitrate

Similar tables have also been published previously (e.g., Betke *et al*, 1967; Cappellini & Fiorelli, 2008; Luzzatto, 2012). The *Predictable Haemolysis* column includes all 7 drugs listed in a recent evidence-based review (Youngster *et al*, 2010). We consider that even a single case of AHA must be taken seriously if the clinical picture is well documented and if AHA is unlikely to have had another cause (e.g. infection): therefore, in addition to those seven drugs, we have retained in this column the following. (a) *Cotrimoxazole*. This drug is used widely in patients with human immunodeficiency virus (HIV) and in other conditions. AHA developed in at least two HIV-acquired immunodeficiency syndrome patients (Tungsiripat *et al*, 2008), one of whom was G6PD-deficient. Additional cases have been reported (reviewed in Ho & Juurlink, 2011). It is presumed that, of the two chemicals present in cotrimoxazole, sulfamethoxazole rather than trimethoprim is likely to be the culprit. (b) *Sulfadiazine*: we found at least one case report (Eldad *et al*, 1991) entirely convincing. (c) *Quinolones*. Although some cases may be regarded as anecdotal, in our view there are at least three convincing ones: one with nalidixic acid (Alessio & Morselli, 1972), one with ciprofloxacin (Sansone *et al*, 2010), and one with ofloxacin (Carmoi *et al*, 2009). Interestingly, the last two patients were (presumably heterozygous) women; the first was unique because she had no illness, but was exposed to nalidixic acid by virtue of working in a chemical factory that produced it. We also feel that if several reports are about chemically related compounds they tend to strengthen each other. The *Possible Haemolysis* column is based on older literature (see for instance Burka *et al*, 1966; WHO Working Group, 1989). As examples of the causal role of some of these drugs in causing AHA in G6PD-deficient persons see Sicard *et al* (1978) for chloroquine, Meloni *et al* (1989) for aspirin, Mehta *et al* (1990) for ascorbic acid, Meloni and Meloni (1996) for glibenclamide.

likely to have been triggered by the infection that had caused them to be prescribed [for instance, AHA is common in G6PD-deficient patients with lobar pneumonia (Tugwell, 1973)].

A recent review (Youngster *et al*, 2010) described a detailed systematic analysis of the literature and concluded that, for only seven drugs there was solid evidence that they cause AHA in G6PD-deficient subjects. We think that the principles of evidence-based medicine serve well the cause of establishing the efficacy of drugs, but they are more difficult to apply to the side-effects of drugs, where even an isolated case report, if well documented, must be taken seriously. Therefore our list is a bit longer, and we have preferred to classify the effect into two groups, where *predictable haemolysis* means that AHA can be expected in a G6PD-deficient patient, whereas *possible haemolysis* means that AHA may or may not take place, depending on dosage, other concomitant drugs, co-morbidity and other factors. Thus, the list in Table I is similar to that in the current *British National Formulary* (www.bnf.org).

Methylene blue (MB, also termed methylthioninium chloride) deserves special mention for two reasons. First, it is used for the treatment of methaemoglobinemia, where it enhances reversion of methaemoglobin back to haemoglobin via NADPH-dependent methaemoglobin reductase. However, if a G6PD-deficient patient has acquired methaemoglobinaemia induced by drugs or other agents, MB will only make things worse by causing AHA (Gauthier, 2000; Liao *et al*, 2002; Foltz *et al*, 2006; Brewer, 2007). Second, MB has been recently revived as an antimalarial (in combination with artemisinin-based combinations) because it has gametocytocidal action against *P. Falciparum*. This is of considerable interest (see section on primaquine below): however, MB at 15 mg/kg/day has been associated with significant haemolysis in G6PD-deficient children in Burkina Faso (Muller *et al*, 2013). It remains to be seen whether at this or lower dosage the benefits may outweigh this side effects in any individual community.

The case of rasburicase (urate oxidase)

This drug, licensed about 10 years ago for the prohpylaxis and treatment of hyperuricaemia associated with tumour lysis syndrome (TLS), deserves special attention for several reasons. First, with other drugs the precise chemical mecha-

nism whereby they cause oxidative damage is not fully understood: for rasburicase – a genetically engineered form of the enzyme urate oxidase – we know instead that, like all other oxidases, one of the products of the enzyme reaction is hydrogen peroxide, of which one molecule is produced stoichiometrically for every molecule of uric acid that is catabolized. In normal cells, hydrogen peroxide is promptly degraded by either glutathione peroxidase (GSHPX) or catalase, but in red cells of G6PD-deficient subjects, GSHPX activity is impaired because GSH is in short supply (Fig 4), and catalase may also be impaired because the intracellular NADPH concentration is low (Gaetani *et al*, 1994, 1996). Second, presumably as a result of this mechanism, in G6PD-deficient subjects rasburicase causes both AHA and acute methaemoglobinaemia – the latter can reach levels of up to 20%, much higher than that seen with other drugs. This literally colours the clinical picture, because the patient appears cyanotic; and methaemoglobinaemia makes the tissue hypoxia consequent on anaemia more severe, because methaemoglobin does not carry oxygen. Third, rasburicase is used in patients who have malignancies (such as acute leukaemia with high blast counts, large cell lymphoma, and sometimes other solid tumours), and who, following chemotherapy, may develop TLS. This means that the drug is administered to a patient who is already quite ill, may be already anaemic, and may be already at risk of renal failure. In addition, rasburicase has a $t_{1/2}$ of 18–24 h resulting in the production of hydrogen peroxide for several days. Fourth, the association between rasburicase-induced AHA and G6PD deficiency is so strong, that this is formally stated as a contra-indication. In a recent compilation of case reports (Sonbol *et al*, 2013), three patients were listed as G6PD normal, but in fact in one of them the test was not done (Kizer *et al*, 2006); in two no quantitative results were given (Kizer *et al*, 2006; Bauters *et al*, 2011), and the test was carried out after a haemolytic attack (when a false-normal result is possible), and after blood transfusion: therefore the G6PD status must be regarded as doubtful (the enzyme test ought to have been repeated subsequently, or a molecular test should have been done).

In an authoritative paper on the evidence-based management of TLS, rasburicase is said to be 'contraindicated in patients with a history consistent with glucose-6 phosphate dehydrogenase' (Cairo *et al*, 2010). Leaving aside the typographical error whereby the word deficiency was omitted, this suggestion is rather strange, since the majority of patients with G6PD deficiency have never had a haemolytic attack, and therefore they or their relatives will provide no such history. The recommendation must be instead to do a G6PD test before giving rasburicase, but a recurrent statement in the relevant literature is that under the pressure of impending TLS there is no time to wait for the result. In fact a G6PD test can be done in 20 min and, were it not outsourced, the result should be available within 1 h. It seems to us incongruous that in highly specialized facilities where, for instance, arterial blood gases and flow cytometry are routine, one cannot do a simple G6PD test (even a 'screening test' would be much better than nothing, see section below on G6PD testing), costing a small fraction of a single dose of rasburicase. No clinical unit treating acute leukaemia or lymphoma should be accredited if a G6PD test result cannot be obtained promptly. The same ought to apply to neonatal units where rasburicase is now used for 'acute kidney injury' (Poliseno *et al*, 2010): in a G6PD-deficient baby, who had not been tested, the result was fatal (Zaramella *et al*, 2013).

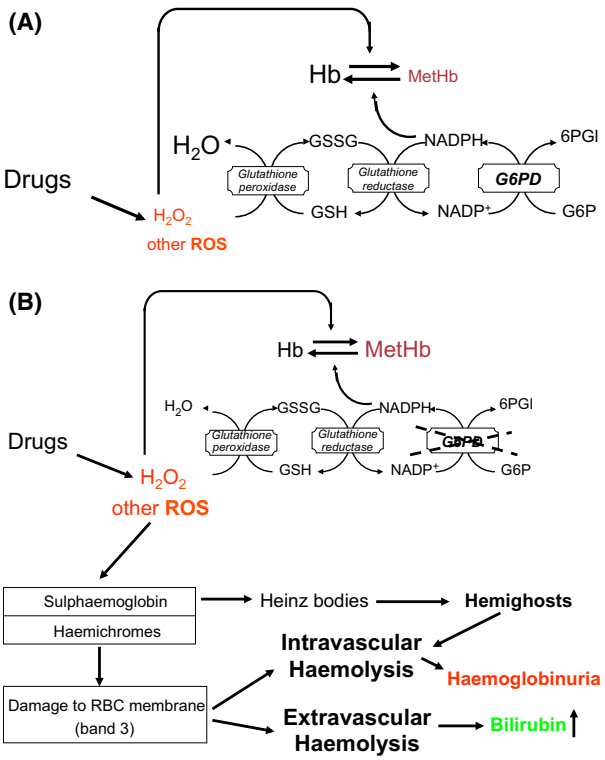

Fig. 4. Diagram showing red cell response to oxidative damage from drugs. (A). In glucose 6-phosphate dehydrogenase (G6PD) normal red cells, hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) are detoxified by glutathione (GSH) peroxidase, which ultimately depends on G6PD activity for the continued regeneration of GSH (for simplicity, the role of catalase is not shown). Also, there is no significant accumulation of methaemoglobin (MetHb) because NADPH-dependent methaemoglobin reductase comes into play, backing up the NADH-dependent methaemoglobin reductase (not shown) that operates in red cells all the time. (B). When G6PD-deficient red cells are exposed to an oxidative challenge GSH will be rapidly exhausted. As a result, H_2O_2 and other ROS are not detoxified: methaemoglobin is allowed to build up and, more seriously, sulphydryl groups in haemoglobin are attacked, resulting in the formation of Heinz bodies, damage to the membrane and, eventually, the destruction of red cells through both intravascular and extravascular mechanisms. It is not clear why with some drugs there is more methaemoglobinaemia than with others: the case of rasburicase (see text) suggests that this may have to do specifically with H_2O_2. GSSG, glutathione disulfide; NADP(H), nicotinamide adenine dinucleotide phosphate (reduced form); 6PGI, glucose 6-phosphate isomerase; G6P, glucose 6-phosphate.

Once again we must heed the principles of pharmacogenetics. The full prescribing information for rasburicase states that 'the incidence of anaphylaxis, hemolysis, and methemoglobinemia was <1% of the 887 rasburicase-treated patients entered on these clinical trials' (see http://products.sanofi.us/elitek/elitek.html#section-9). This is not surprising, as it reflects the frequency of G6PD deficiency in a random group of patients; but it is also misleading, because the effect of the drug in any G6PD-deficient patient is serious and predictable. A separate issue is whether in a certain clinical situation, with the full knowledge that a patient is G6PD-deficient, one may administer rasburicase nevertheless, having deliberated that allopurinol is not a valid alternative, and having weighed that the life of the patient is more at risk from TLS than from AHA and methaemoglobinaemia, which will develop and will be appropriately managed.

Management and Prevention of G6PD-related AHA

Treatment of AHA

Making a diagnosis of G6PD-related AHA has been reviewed elsewhere (Luzzatto, 2012) and is therefore not discussed here: probably the most important limiting factor is to think about it. Once the diagnosis is made and the offending drug is discontinued, all that is needed in most cases is to supervise recovery, which will take place on its own without any specific treatment. However, there are two important exceptions.

1 If the anaemia is very severe, either because there was pre-existing anaemia or because the fall in Hb caused by the drug has been massive, then prompt blood transfusion may be required to save the patient's life. Generally speaking, this situation is more dangerous in children. There are no evidence-based guidelines, but the following guidance may be helpful.
   - If the haemoglobin level is below 70 g/l, proceed with blood transfusion
   - If the haemoglobin level is below 90 g/l and there is evidence of persistent brisk haemolysis (haemoglobinuria), immediate blood transfusion is also indicated.
   - If the haemoglobin level is between 70 and 90 g/l but there is no haemoglobinuria this means probably that AHA is subsiding: blood transfusion can be probably withheld provided the patient is observed closely for at least 48 h.
2 In the context of severe AHA, acute renal failure (ARF) may develop. This is not due to haemoglobinuria causing damage to normal kidneys, but rather to a situation similar to hypovolaemic shock. This complication is generally rare in children [although in one series it occurred in up to one-third of cases: (Balaka *et al*, 2003)], but less rare in adults, and is probably more likely if there was some pre-existing renal damage. Haemodialysis may be required in some cases, but renal function will usually recover.

G6PD testing

In general, a test for G6PD deficiency is required for one of two purposes. (i) As a diagnostic test when G6PD-related AHA is suspected. (ii) As a preventive measure when considering administration of a drug that is potentially haemolytic for G6PD-deficient subjects. Item (i) is outside the scope of this review; item (ii) is not simple. To request a G6PD test whenever a drug listed in Table I is prescribed would be probably asking too much; and if a person is already known to be G6PD-deficient the test is unnecessary. On the other hand, there are at least two circumstances when G6PD testing, in our view, ought to be compulsory. (i) When a potentially haemolytic drug is given in an area where G6PD deficiency is known to be common, and more so in the context of MDA: the obvious example being PQ. (ii) When a particularly dangerous drug is given in an already compromised clinical situation: the example of rasburicase has been discussed above in some detail.

The gold standard for measuring G6PD activity is the quantitative spectrophotometric assay. In normal red cells from normal subjects, G6PD activity, when measured at 30°C, ranges from 7 to 10 iu/g Hb [(Dacie & Lewis, 1995): but many laboratories give wider reference values, e.g. from 4 to 12 iu/g Hb]. In most G6PD-deficient persons in the steady state, the red cell G6PD activity will be <20% of normal, or below 2 iu/g Hb (the activity will be higher when there is haemolysis; see below),

Leaving aside technical issues (e.g. appropriate collection/storage of samples), the results of G6PD testing in males is almost always straightforward: there is a sharp demarcation between G6PD normal and G6PD-deficient samples. This has made it possible to adopt 'screening tests' much simpler than the spectrophotometric assay. The first such methods were the dye decolourization test (Motulsky & Campbell-Kraut, 1961) and the methaemoglobin reduction test (Brewer *et al*, 1962): although they proved quite reliable and suitable for testing large numbers of samples, they are hardly used any more because they have never been made into commercial products. There are currently two commercially available rapid diagnostic tests (RDT): a fluorescence spot test (Beutler *et al*, 1979), and a formazan-based spot test (Tantular & Kawamoto, 2003). To carry out the former an ultraviolet lamp is needed; for the latter, no equipment is needed whatsoever. Therefore these tests are eminently suitable for field work and for 'point of care' use: the only limiting factors for large scale use being the cost and proper storage of the reagent kits.

Unlike in subjects who are clinically normal at the time of testing, in a patient with AHA the pitfall in the laboratory diagnosis of G6PD deficiency is that, as a result of the selective destruction of the older, more deficient red cells coupled with the entry in circulation of numerous reticulocytes, there

will be a transient substantial increase in G6PD activity. The extent of this increase will vary with different G6PD variants: but the fact remains that the patient is being tested at the very time when the chances of mis-classifying a G6PD-deficient person as G6PD normal is highest, and this is true not only for RDTs but even for the spectrophotometric assay: therefore, a normal result ought to be re-checked about 2 months later.

Heterozygotes for G6PD deficiency

In females, by virtue of the X chromosome inactivation phenomenon, the distribution of G6PD activity values is a continuum, whereby heterozygotes have a wide range, with values overlapping at one end with G6PD normal homozygotes and at the other end with G6PD-deficient homozygotes (Nance, 1964; Rinaldi *et al*, 1976). As a result, the heterozygous state can be identified with confidence only in the middle range, say when the spectrophotometrically measured G6PD activity is between 30 and 70% of normal: in all other cases we remain uncertain between heterozygous and homozygous normal or between heterozygous and homozygous deficient. This also means that RDTs will often yield an intermediate result, which must be regarded as 'doubtful'. As this problem has a biological rather than a technical basis, in many cases the identification of heterozygotes can be rigorously established only be molecular analysis (see below). However, for the important purpose of assessing the G6PD status before administering a potentially haemolytic drug such as PQ, the situation is not as problematic as it might seem. Indeed, if a heterozygote female tests as G6PD-deficient, in practice her AHA is likely to be just as severe as in a G6PD-deficient male, whereas a normal G6PD result indicates that she is likely not to develop clinically significant AHA, and a result in the middle range indicates that her AHA is likely to be mild.

Molecular analysis

Currently, mutation analysis at the DNA level is easy: it can be called a genotypic analysis (as opposed to the phenotypic analysis based on enzyme activity), and G6PD testing by this approach is attractive (Poggi *et al*, 1990; Minucci *et al*, 2012b). In many populations one or more G6PD deficiency alleles are well known: for example, in Africa there are 3 mutations underlying the A- variant of G6PD (Beutler *et al*, 1989); in Sardinia the large majority of G6PD-deficient people have the G6PD Mediterranean variant (Cappellini *et al*, 1996), but some have G6PD Seattle (Frigerio *et al*, 1994). A DNA-based approach is fine as long as a known mutation is found; however, if it is not found it may be that the patient in question has an unexpected mutation (in which case it would be unsafe to presume that the patient is G6PD normal), and therefore in such cases a phenotype-based approach is mandatory. Of course it may not be long before complete sequencing of all exons and intron-exon junctions may be sufficiently cheap to become routine. For the time being the main asset of molecular analysis is that, for instance, in studies of population genetics all heterozygotes can be detected unambiguously, once the polymorphic variants in that population are known. From the clinical point of view, instead, we must remember that, as outlined in the section above, heterozygotes may or may not be at risk of severe AHA: in this respect the certain detection of every heterozygote is not necessarily an advantage over knowing what proportion of an individual heterozygote's red cells have normal G6PD activity. In other words, from the practical point, i.e. in terms of clinical implications, the phenotypic expression is not surprisingly more important than the genotypic classification.

Tests for drugs that may cause haemolysis

As mentioned above, PQ or dapsone do not cause haemolysis of G6PD-deficient red cells *in vitro*: either the active molecule is a metabolite of PQ, or additional factors are required that have not been provided *in vitro*, but are present *in vivo*. Attempts have been made to obtain and test metabolites by incubating the drug with liver microsomes (Bloom *et al*, 1983); and drugs have been tested *in vitro* for their ability to stimulate the pentose phosphate pathway (Gaetani *et al*, 1976), since this would be expected to correlate with haemolysis *in vivo*. However, neither of these approaches has been systematically followed up. We must admit that, to date, any new licensed drug has not been tested for its potential to cause AHA in G6PD-deficient subjects.

Animal models

Exploring animal models has been in the best tradition of physiology and pathophysiology for a long time. Nowadays in many cases these can be produced by genetic engineering; and, in a pharmacogenetic setting such as G6PD, an animal model would be especially valuable as a test system. A targeted 'knock-out' of the *G6PD* gene in mouse was found to be an embryonic lethal (Longo *et al*, 2002), thus yielding no model. A G6PD-deficient mouse obtained by mutagenesis (Pretsch *et al*, 1988) – with a level of G6PD activity in red cells that is about 20% of that in red cells of a normal mouse AHA (Neifer *et al*, 1991) – is viable. The mouse has a splicing mutation in the *G6PD* gene (Sanders *et al*, 1997), and it has been used extensively to investigate the possible role of G6PD with respect to cardiovascular disorders (Matsui *et al*, 2006). It appeared that this mutant mouse did not develop drug-induced AHA [(personal communication from the late Ulrich Bienzle of the Institute of Tropical Medicine, Berlin)]; however, very recently Zhang *et al* (2013) have achieved AHA in these mice by administering PQ, although at much higher doses (per body weight) than are used therapeutically in humans. Thus, it appears that an outright knock-out of *G6PD* is too drastic, whereas in the available mutant mice G6PD

deficiency is rather mild. In future, one might take advantage of the high sequence conservation of this gene from mouse to human (94%), in order to try and 'knock-in' a human mutation that gives no clinical phenotype until challenged by PQ, i.e. any of those present in known polymorphic mutants.

Very recently, G6PD deficiency has been produced in zebrafish embryos by the morpholino technique (Patrinostro *et al*, 2013). Particularly relevant to testing new drugs, morpholino-inhibition of G6PD synthesis has been titrated to a level at which there was no anaemia in the absence of challenge, but severe haemolytic anaemia developed when the embryos were exposed to primaquine.

The current resurgence of primaquine

For some decades most of the limelight in the malaria field has been on the treatment of acute *P. falciparum* malaria, because this condition is highly lethal, especially in children. PQ has not been prominent in this respect; in most endemic areas chloroquine was the mainstay of treatment until the 1980s, when resistance to this agent as well as to sulfadoxine-pyrimethamine unfortunately became widespread (Yeung *et al*, 2004). Subsequently, several alternatives have been employed, and artemisinin-containing drug combinations are currently the recommended standard (WHO, 2010). Several drugs are also available for the treatment of *Plasmodium vivax* malaria (WHO, 2010). However, there are two specific indications for the use of PQ. (i) With respect to *P. falciparum,* for the purpose of eliminating gametocytes. Standard drug regimens are effective against the asexual parasites that are responsible for all clinical manifestations; but becasue they spare gametocytes, the clinically cured patient remains infectious to mosquitoes and through them can transmit malaria to others. (ii) In cases of *P. vivax*, for the purpose of preventing relapse. Unlike with *P. falciparum*, where recurrence is always due to re-infection, with *P. vivax* there is long-term persistence in the liver of a special form of the parasite called a hypnozoite. Remarkably, PQ is still the *only* effective drug in use today for these two indications. Tafenoquine, currently under experimentation, may be an alternative, but it is already known that this drug can be potently haemolytic for G6PD-deficient patients (Shanks *et al*, 2001).

Because G6PD deficiency is notoriously common in areas of endemic malaria (Howes *et al*, 2012), WHO recommends testing for this before giving PQ; in practice, however, this is rarely done. In many cases there is rather a tendency, whenever possible, to treat malaria with other drugs, and to withhold PQ in the interest of safety. However, an increasing number of countries in Asia have set or will set themselves the ambitious goal to eliminate *P. vivax* malaria: in order to do this, PQ is needed. In addition, in areas where artemisinin-resistance is beginning to occur it is especially important to prevent transmission of *P. falciparum*, and again, in order to achieve this the gametocytocidal effect of PQ is needed (White *et al*, 2012).

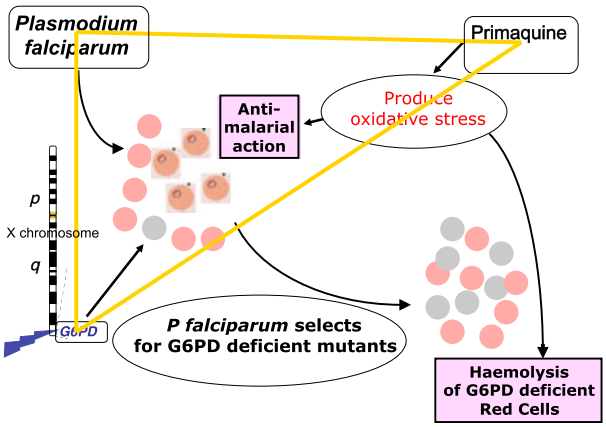

Fig. 5. The triangular relationship between primaquine, G6PD and *Plasmodium falciparum*. It is intriguing that *P. falciparum* has been a major selective force in increasing the frequency of glucose 6-phosphate dehydrogenase (G6PD) deficiency (Luzzatto, 1979), that primaquine is a powerful agent against *P. falciparum* gametocytes, but when thus used it causes AHA in G6PD-deficient persons. The simplest explanation is that the same oxidative stress is damaging for both the parasites and the G6PD-deficient red cells. Methylene blue, like primaquine, is also gametocytocidal and haemolytic in G6PD-deficient persons (see text). Fortunately, this is not so for other antimalarials that have different mechanisms of action, but, unfortunately, these are not gametocytocidal.

In these situations the clash between PQ and G6PD deficiency has escalated from a circumscribed pharmacogenetic problem to a major public health issue. In principle, there are two solutions. (i) To give PQ regardless, and let the G6PD-deficient patients bear the consequences, with the hope that appropriate medical supervision and intervention will be available when necessary. (ii) To test for G6PD, and then either exempt those who are G6PD-deficient from receiving PQ, or give them PQ under supervision. Solution (ii) has been regarded hitherto as impractical, but fortunately in recent times there has been some deliberate effort to make G6PD testing easier and more affordable: indeed, a formazan-based RDT has been field-tested (Kawamoto *et al*, 2006). In the meantime, WHO has very recently recommended an important change for one of the two indications for PQ mentioned above: for preventing transmission of *P. falciparum* the recommended dose has been decreased from 0·75 mg/kg (adult dose 45 mg once only) to 0·25 mg/kg (adult dose 15 mg once only). This seems a good compromise as there is evidence that the gametocytocidal action may be sufficient, and the AHA caused in G6PD-deficient patients will be certainly much milder (White *et al*, 2012).

Conclusion

Some 80 years since haemolytic complications from 8-aminoquinolines were first observed, AHA in G6PD-deficient persons remains a unique case in pharmacogenetics, where a specific enzyme deficiency is the single determinant of a severe, potentially life-threatening side effect. Unlike with

other enzyme-related idiosyncrasies, such as those associated with porphyrias (Thunell *et al*, 2007), where the condition is rare and there are usually alternatives to the culprit drugs, G6PD deficiency is common in many populations, and it is common precisely where drugs such as PQ are needed. The intriguing triangular relationship (see Fig 5) – whereby malaria selects for G6PD deficiency, PQ is an effective antimalarial, but G6PD-deficient persons are so sensitive to PQ, has been discussed elsewhere (Luzzatto, 2010). Suffice it to say here that, as a result, drug-induced AHA is both a clinical and a public health problem.

Although they are now rather well identified and defined, both of these problems are still challenging. On the clinical side, G6PD deficiency must be always considered in the differential diagnosis of haemolytic anaemia; and we should be mindful of G6PD deficiency whenever we prescribe a potentially haemolytic drug. On the public health side, it is no longer permissible – not that we think it ever was – to sweep the issue under the carpet when administering primaquine or dapsone widely in areas where G6PD deficiency has a high prevalence: until alternative drugs become available, G6PD testing must be made available.

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