Iron and infection: effects of host iron status and the iron-regulatory genes haptoglobin and NRAMP1 (SLC11A1) on host–pathogen interactions in tuberculosis and HIV

Joann M. McDERMID* and Andrew M. PRENTICE*†

* MRC International Nutrition Group, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K., and † MRC Keneba, The Gambia, West Africa

ABSTRACT

There are many lines of evidence illustrating that iron plays a pivotal role in modulating the battle for survival between mammalian hosts and their pathogens. Each displays considerable genetic investment in a wide range of mechanisms for acquiring and maintaining iron. These competitive mechanisms are highly complex, existing within an interacting matrix of absorption, transport, storage and detoxification systems, each of which are iron-responsive and thus able to adapt to the different phases of infection. Considerable genetic polymorphism in some of these systems, with signals of geographic selection in the hosts, and niche selection in the pathogens, indicates that they are critical for species survival. In this review we briefly summarize the role of iron in host immune function before reviewing the available evidence that iron modulates susceptibility and disease outcomes in HIV and TB (tuberculosis). We then examine the putative role of iron-related host genes by focussing on two candidate genes, haptoglobin and NRAMP1, for which there are common polymorphic variants in humans with strong evidence of functionally distinct biochemical phenotypes that would be predicted to influence the course of HIV and TB infections. Finally, we examine the limited evidence so far available that nutrient–gene interactions are likely to influence the way in which gene variants can protect against infection. We conclude that there is a wealth of evidence associating alterations in iron balance and in iron-regulatory systems with disease progression, but that many issues related to the direction of causality, mechanisms of action and sensitivity to pharmacological intervention remain to be elucidated. Since iron is probably the most widely prescribed compound throughout the world, used in both preventative and treatment regimens, a deeper understanding of the host–pathogen interactions relating to iron constitutes an important area for both basic and clinical research.

Key words: haptoglobin, HIV, iron, infection, NRAMP, pathogen, tuberculosis.

Abbreviations: APR, acute-phase response; AZT, azidothymidine; CI, confidence interval; CQ, chloroquine; DFX, desferrioxamine; Hp, haptoglobin; HR, hazard ratio; IFNy, interferon γ; IL, interleukin; LD, linkage disequilibrium; LPS, lipopolysaccharide; Mtb, Mycobacterium tuberculosis; NF-κB, nuclear factor κB; NO, nitric oxide; NTBI, non-Tf-bound iron; PCP, Pneumocystis carinii pneumonia; PTB, pulmonary TB; RES, reticulo-endothelial system; ROS, reactive oxygen species; TB, tuberculosis; Tf, transferrin; TfR, Tf receptor; TNFa, tumour necrosis factor α.

Note: it is customary to label the murine version as Nramp and the human version as NRAMP but, as we frequently use the term as a general descriptor, we will use NRAMP throughout. Italics represent the gene, and plain text represents the protein product.

Correspondence: Professor Andrew M. Prentice, MRC International Nutrition Group, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K. (email andrew.prentice@lshtm.ac.uk).

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INTRODUCTION

Among all the nutrients, iron stands out as having a particularly crucial role in mediating host–pathogen interactions. In animals, evidence for this comes from the diversity of their methods of iron sequestration and from their ability to further deprive invading organisms of iron by depleting plasma iron during the APR (acute-phase response). In pathogens, the strongest evidence comes from the high degree of genomic investment in systems for iron acquisition, the vast range of such systems and their very low iron-binding constants which permit them to be effective even in highly iron-depleted environments [1]. At first sight the suggestion that iron lies at the centre of an eons-long battle between hosts and their pathogens is surprising, since iron is the second most abundant element on the earth’s crust. The explanation lies in a combination of the extreme usefulness of the redox potentials of the ferrous (Fe^{2+})/ferric (Fe^{3+}) switch (which makes it widely used in enzymic and signalling systems), and the fact that most iron exists in a highly insoluble oxidised state.

In this review, we summarize current knowledge on the relationships between iron and two of the leading infectious causes of death in humankind: HIV and TB (tuberculosis). We examine the relationships both in terms of cause (i.e. the question of whether positive or negative perturbations in iron status affect susceptibility to disease and clinical progression), and in terms of effect (i.e. the pathological consequences of infection on iron homeostasis). We also examine whether variants in host genes may influence these outcomes. At present, the evidence for gene–nutrient interactions is somewhat limited, so we focus on just two candidate genes that have been examined in some detail: the haemoglobin-binding acute-phase protein, Hp (haptoglobin), and the divalent-cation transporter from the phagosome membrane of the macrophage NRAMP1 [natural-resistance-associated macrophage protein-1; now known as SLC11A1 (solute carrier family 11, member 1)].

IRON AND IMMUNE FUNCTION

As iron is such a ubiquitous element in biological systems it is no surprise that it is intimately involved in many facets of immunity [2–5]. For the purposes of this review, the key question is: which of these processes is impaired within the range of deviations in iron homeostasis seen in iron deficiency or in iron overload? There is a large, and often contradictory, body of literature on this topic with more evidence drawn from in vitro and experimental animal studies than from direct examination in humans. Table 1 summarizes this evidence in the broadest of terms.

We have argued elsewhere [2] that there may be a relatively narrow range of host iron required for optimal defence against pathogens. This argument is summarized in Figure 1, which shows how detrimental effects in the human host of either deficiency or excess will interplay with similar benefits and deficits for a given pathogen, and that these will differ across individuals and pathogens according to their specific genetic inheritance. Several further inferences can be drawn.

First, that the optimal level of iron status for host defence is likely to differ from the optimal level for other physiological functions, such as erythropoiesis, work performance or cognitive function. This leads to the proposition that a degree of iron deficiency (as seen in most poor communities in the developing world) may have had adaptive value in protecting from infective disease; a view considered heretical by the proponents of mass iron supplementation. There is a body of evidence to support this controversial view, and some cautionary considerations to be drawn from a number of iron-supplementation programmes, some examples of which are outlined below.

Secondly, that the optimal window of host iron status may differ depending on the pathogen and across the
Figure 1 Possible effects of iron in mediating host–pathogen interactions

DYSREGULATION OF IRON METABOLISM IN DISEASE

Alterations in iron metabolism during infections can occur as an intended component of the series of cytokine-orchestrated defensive strategies of the APR or as an unintended consequence that often advances the pathology and can increase mortality. The former includes alterations in iron distribution mediated through changes in the primary iron and haem transporters and storage molecules [Tf (transferrin), ferritin, lactoferrin, Hp, haemopexin and caeruloplasmin] [2], and a reduction in intestinal iron absorption almost certainly regulated by the newly discovered acute-phase protein, hepcidin [16–18]. The unintended consequences most notably include ACD (anaemia of chronic disease) in which cytokine blockade of iron redistribution can yield the co-existence of peripheral anaemia and iron-overload in the liver and/or bone marrow [19].

IRON AND HIV INFECTION

Evidence of altered iron metabolism in HIV
HIV infection can cause the paradoxical alterations in iron metabolism alluded to above with the co-existence of anaemia alongside high circulating ferritin and iron deposits suggestive of iron overload. Since many of the studies reporting abnormal iron status have been conducted in individuals with advanced HIV infection, it is

various bodily niches in which pathogens can establish a foothold. For example, the gut pathogen Yersinia enterolytica cannot survive in the low-iron environment of plasma, but Y. pestis has managed to establish itself as a systemic infection through, among other adaptations, the evolutionary acquisition of a number of iron-transport genes by horizontal capture from another bacterium [14]. Similarly, only certain variants of Escherichia coli, characterized by iron transporters in the pathogenicity-determining regions of their genome, can colonize the very low iron environment of the urinary tract (for example, [15]).

Thirdly, that since different pathogens (and, as shown above for E. coli, even different strains within species) will be able to multiply at different iron concentrations, the host has to compromise in selecting the optimal balance that protects its own physiological function whilst defending against the broadest possible spectrum of infections.

Fourthly, that variations in the host genome affecting iron metabolism may reach an equilibrium within populations due to balancing protection against different organisms and may become deleterious in the absence of those infections. Furthermore, these variations may have different consequences depending on the prevailing iron content of the diet and other factors affecting iron status (e.g. helminth infections).

Understanding these nutrient–gene interactions will be one of the major challenges of the post-genome era. We will discuss this below in terms of NRAMP and mycobacterial infections.
difficult to determine the temporal sequence of these alterations which might be due to HIV infection itself, to opportunistic infections and their complications or to treatments (e.g. blood transfusions or marrow-toxic zidovudine [AZT (azidothymidine)]) that influence iron status in HIV infection [20–24]. It is possible that aberrations may have begun early in infection and preceded the development of clinical symptoms. Spada et al. [25] found that, although the degree of impairment of haematopoiesis (marked by decreased serum iron and haemoglobin) was greater with more advanced disease, aberrations were also observed early in the disease process.

Altered iron metabolism in HIV infection may present as hypoferraemia, hypotransferrinaemia, low or high Tf saturation and moderate to severe hyperferrinaemia combined with increased deposition of iron ferritin and/or haemosiderin in macrophages, microglia, Kupffer cells, endothelial cells and myocytes of the bone marrow, brain white matter, skeletal muscle and sometimes liver [26,27]. The possible aetiology of these changes is complicated and not yet fully elucidated but, as bone marrow iron stores are often increased, it suggests that iron metabolism in HIV may be affected by a functional block of iron release from the RES (reticulo-endothelial system) [28]. Karcher and Frost [29] reported that RES blockage and excess macrophage iron were observed in 65% of AIDS subjects, while iron stores were absent in only 16% of subjects. In a study from early in the epidemic (1984–1987), 155 subjects were recruited to examine peripheral blood and bone marrow aspirates [23]. The majority of subjects were experiencing iron overload (93% in those on AZT therapy compared with 65% in the remainder), whereas only 2% had iron deficiency. Thus decreased serum iron levels could represent immunologically altered iron metabolism rather than true iron deficiency. This is supported by the observation that clinically apparent iron deficiency is rarely improved by oral iron supplementation [28].

**Consequences of HIV-associated anaemia**

Anaemia is the most commonly reported haematological disturbance in HIV infection with prevalence estimates ranging from 10–95% in different clinical settings and populations [30–33]. Epidemiological studies of HIV-related anaemia have demonstrated repeatedly strong associations between low haemoglobin concentrations and disease progression and mortality (Table 2) [31,34,35]. Those at greater risk for anaemia are of African–American descent, with low CD4+ count, high viral load, low mean corpuscular volume and those receiving AZT [36]. Although the association between anaemia and decreased survival has not been proven to be causal, the consistent findings of numerous studies of large observational cohort design suggest anaemia is an important factor in understanding the clinical course of subjects with HIV.

![Table 2: HIV-associated anaemia and survival](image-url)
Iron and infection: effects of host iron status and iron-regulatory genes

Not only is anaemia associated with a poorer prognosis, but haematological recovery is associated with an improved prognosis and better quality of life [37]. Treatment of anaemia was associated with improved survival times over a range of CD4 counts; however, some data suggest that caution is warranted in the management of anaemia. Blood transfusions were problematic and associated with accelerated mortality, even early in the course of HIV infection [35]. This could be secondary to transfusion-transmitted infection, transfusion-related immunosuppression (an immunomodulatory effect of up-regulating humoral immunity and down-regulating cell-mediated immunity) [38–41] or possible transient activation of HIV expression, which may provide an opportunity for developing viral resistance if concurrently using antiretrovirals.

Consequences of elevated iron in HIV infection

An early study by Fuchs et al. [42] demonstrated that ferritin was negatively correlated with haemoglobin and positively correlated with neopterin, a marker of both macrophage activation and HIV disease progression. They observed that increasing concentrations of ferritin were accompanied, or closely preceded, by rapid disease progression in HIV-seropositive children. Other studies have shown that serum ferritin concentrations in HIV-seropositive subjects were associated with the severity of infection when evaluated clinically as disease stage, immunologically as CD4+ cell count or virologically as serum p24 antigen titre and plasma viral burden [22,43–46].

Given these early indications of a role for elevated iron in vivo, mechanisms that might explain the possible detrimental effect of iron have been subsequently investigated in HIV infection (reviewed in [26,27]). These include: direct cytotoxicity and immune dysfunction; enhancement of viral replication; predisposition to certain opportunistic infections and neoplasia; and alterations in the immune response that may affect HIV virulence.

Iron loading is associated with the active production of hydroxyl radicals through the Haber–Weiss or Fenton reaction. The resulting ROS (reactive oxygen species) are associated with the oxidation of nucleic acids, chromosome breaks and peroxidation of unsaturated fatty acids. It is possible for limited chromosomal damage to be repaired; however, extensive DNA damage promotes apoptosis of the affected cell. A number of studies have shown that ROS may play a role in HIV progression by induction of apoptosis, with the ensuing depletion of CD4+ cells [47–50]. Antioxidants may inhibit apoptosis in vitro [51–53] and N-acetylcysteine was demonstrated to have this effect in vitro in a small observational study with 15 subjects over the course of 6 months [54]. Chronic oxidative stress has also been linked hypothetically with abnormal immune function, particularly T-cell function (reviewed in [51,55]). In vitro findings suggest the involvement of ROS in signal transduction pathways leading to the activation of NF-κB (nuclear factor κB) which potently increases the production of HIV virions in latently infected cells [50,51,56].

Given that ROS probably promotes HIV transcription, it is important to consider if antioxidants are associated with a reduction in HIV viral replication, or conversely, if iron supplementation is associated with enhanced viral load. A number of studies in the early 1990s have shown a reduction in virus replication associated with antioxidant supplementation in vitro (primarily using non-nutrient antioxidants, with the exception of ascorbate); however, human studies have not been able to consistently replicate these effects [57–59].

Very limited research has been published examining the relationship between iron supplementation and HIV viral load. A recent report of a randomized placebo-controlled double-blind clinical trial from Kenya did not show enhanced HIV-1 viral load in subjects receiving iron supplements (60 mg of elemental iron, twice weekly, for 4 months) [60]. This dosage, however, is below the standard daily dose for treatment of anaemia and anaemia prevention in pregnant women and, thus, further investigation is required.

An important study by de Monye et al. [61] attempted to overcome the cause-versus-effect conundrum by examining retrospectively whether iron stores from bone marrow aspirates were associated with survival in HIV patients prior to the introduction of HAART (highly active antiretroviral therapy). They observed that rate of death was significantly higher among subjects with the highest grade of iron storage compared with the lowest grade from the time of the bone aspirate {HR (hazard ratio), 2.1 [95 % CI (confidence interval), 1.3–3.5]} and from the time of HIV-seropositivity {HR, 2.8 [95 % CI, 1.4–4.9] after adjustment for absolute lymphocyte count, blood transfusion history, haemoglobin and absolute neutrophil count. They also found that certain infections, including Candida spp., Pneumocystis carinii and Mycobacterium species, were more likely to be reported in medical records for subjects with the highest grade of iron storage compared with those with the lowest grade (P = 0.006). The main drawbacks of this study [61] are that CD4 count was not available for the majority of subjects and that subjects were selected on the basis of having had a bone marrow biopsy, therefore indicating a possible bias as a result of the severity of HIV infection.

The direct effect of iron as a growth factor for specific pathogens causing opportunistic infections in HIV has been investigated, and it has been shown in vitro [62–64] and in murine models [62,65] that iron influences the development of Mycobacterium infections (see below). In vivo evidence for another important HIV-related mortality effect comes from a clinical trial in which...
subjects received either aerosolized pentamidine or oral dapsone (which contained 60 mg of iron as iron protosolate) for PCP (P. carinii pneumonia) interventions [66]. This study was discontinued due to lower CD4+ counts and a higher mortality rate in the dapsone + iron group. The authors speculate that these differences may have been due to the oxidative effect of dapsone or due to the addition of the iron. Given that two independent studies [67,68] were conducted where iron was not part of the dapsone preparation and that these did not indicate significant mortality differences, a detrimental role for iron is likely. This evidence must be viewed as suggestive, rather than conclusive, as the latter studies were conducted among subjects with less advanced HIV infection than the initial study by Salmon-Ceron et al. [66].

In a study by Mateos et al. [69], total iron and the iron-binding proteins Tf, lactoferrin and ferritin were assessed in the acellular bronchoalveolar lavage fluid of HIV-seropositive subjects with PCP and a HIV-seronegative control group (presenting with unexplained dry cough). They observed an 8-fold increase in ferritin and a 6–7-fold increase in total iron concentrations among PCP subjects, but no significant differences in Tf or lactoferrin concentrations. They speculated that the range of ferritin concentrations observed, combined with the lack of increased Tf, is suggestive that the increased iron concentration is due to NTBI (non-Tf-bound iron). NTBI would be highly reactive and induce oxidative stress with potential localized cell damage and, at the same time, increase the iron availability for the growth of P. carinii.

If iron excess is associated with a negative outcome in HIV infection, is iron chelation associated with an improved prognosis? Chelatable iron or ‘free’ iron is a labile iron compartment in dynamic equilibrium with free iron stored in tissues. DFX (desferrioxamine) penetrates monocytes, hepatocytes and myocytes, and targets free iron by forming ferrioxamine, an extremely stable complex that is distributed in the extracellular space and unable to penetrate cells [70]. Therapy with DFX or hydroxypyridine derivatives mobilizes iron not only from the RES, but also from the hepatocellular compartment, and the iron is expelled primarily through urinary excretion.

Iron chelation has been shown to have an effect in vitro on HIV-1 replication. When two HIV-1 provirally infected cell lines were exposed to oxidant stress induced by H2O2, DFX inhibited the activation of NF-κB. This resulted in a significant reduction in p24 antigen production and reverse transcriptase activity in the absence of cytotoxicity [71]. A similar antiretroviral effect was demonstrated using HIV-1-infected peripheral blood mononuclear cells stimulated with IL (interleukin)-2 [71]. DFX, as well as several antioxidants, have been shown by others to block the induction of NF-κB by oxidant stress, and DFX has been shown to protect against TNFα (tumour necrosis factor α)-mediated cytoxicity and HIV-1 replication in other cell lines [72]. However, these findings are not entirely consistent between studies nor are the mechanisms of action certain [73].

A second route by which iron chelation could influence HIV replication is by inhibition of DNA synthesis through inactivation of ribonucleotide reductase. Ribonucleotide reductase inhibition by DFX has been reported by Boelaert et al. [74] and this was accompanied by inhibition of lymphocyte proliferation [70]. Saravino et al. [75] also observed that the expression of TfRs (Tf receptors) is down-modulated by acute HIV-1 infection in T-lymphoid cells and thus cell phenotypic modulation is associated with the cytopathic effects of the virus and, importantly, this effect can be modulated by iron chelation (with sodium citrate or DFX).

Whether the effects of iron chelation are seen in vivo is difficult to confirm. Thalassaemia is the best-studied example where a genetic predisposition to iron overload has been investigated in relation to the possible benefits of iron chelation on HIV progression. In a multicentre cohort study, 64 subjects with thalassaemia and a known date of HIV-1 seroconversion were followed for a median of 6.4 years [76]. Regression models (adjusted for age and splenectomy) revealed that a faster rate of progression to AIDS was associated with lower daily DFX dosages. After following the same subjects (23% died or were censored) for a median of 8.9 years after HIV-1 seroconversion, they reported that increased mean serum ferritin concentrations during follow-up, rather than average daily DFX dosage, were significantly associated with progression to AIDS [HR, 1.44 for each 1000 g/l ferritin increase (95% CI, 1.01–2.07)] and to death [HR, 1.63 for each 1000 g/l ferritin increase (95% CI, 1.10–2.40)] in multivariate regression models adjusted for multiple potential confounders [77].

Finally, an association between iron metabolism and CQ (chloroquine) with anti-HIV activity has been demonstrated in a number of studies. In both iron-loaded (in vivo with iron dextran) and iron-depleted rats, CQ was shown to significantly reduce incorporation of iron into the liver, spleen and alveolar macrophages of animals [78]. Tsai et al. [79] examined the in vitro administration of CQ in a T-cell line and found that it interfered with the post-transcriptional production of HIV-1 virus. This finding was subsequently confirmed in T-cells and monocytes using a CQ analogue, HCQ (hydroxyCQ) [80,81] and in non-laboratory strain cell lines [82], as well as HIV-1 subtype C and HIV-2 [83]. A number of different mechanisms might explain this effect, among them the restriction of intracellular iron that is a necessary cofactor for HIV replication. In two clinical trials, reduced amounts of HIV-1, HIV-1 RNA and IL-6 concentrations were reported in subjects receiving these compounds [84,85].
IRON AND TB

Numerous studies have demonstrated an association between malnutrition and TB (reviewed in [86]); however, there is still limited evidence that isolates the effect of iron. Conversely, there have been significant advances in solving the problems of identifying mechanisms of mycobacterial iron metabolism that were previously too complex to elucidate by conventional biochemical approaches [87]. This has been helped in part by the sequencing of the entire genome of *Mtbc* (*Mycobacterium tuberculosis*) in 1998 [88]. Direct *in vivo* evidence for the dynamic interplay between the human host and *Mtbc* in terms of iron competition during infection and disease outcome, remains limited.

**Host–pathogen iron interactions:** host iron-restriction and *Mtbc* iron acquisition

Given that bacteria require iron to establish infection, propagate pathogenesis and resist host defences, a major component of the host natural immunity is to restrict iron availability to the pathogen. This restriction is characterized by the induction of the host APR that limits the amount of circulating iron, primarily accomplished through a reduction in dietary iron absorption and by iron sequestration. The sequestration of iron by the host also limits iron-induced cellular damage to the host itself through binding by serum proteins such as Tf, ferritin and lactoferrin. Overall, an attempt is made by the host to induce an iron deficiency upon the pathogen.

Indirect evidence of the effect of host iron restriction on the outcome of TB comes from a study by Kochan [89], who demonstrated that Tf was able to inhibit the growth of the tubercle bacillus in human serum in an iron-withholding manner. Later experiments in mice by Lepper et al. [90] extended these preliminary observations by demonstrating a dose–response effect between dietary iron intake and the multiplication of *M. paratuberculosis* in two different murine models leading to greater frequencies of residual and progressive mycobacterial infections. Gomes and co-workers [91] provided supplementary evidence in another *Mycobacterium* species, *M. avium*, of the ability of three different iron chelators to reduce bacterial proliferation *in vitro* in axenic media. Importantly, these investigators found that an iron-reduced diet (leading to severe iron deficiency) had a significantly greater effect in reducing bacterial proliferation in mice than intraperitoneal injection of iron chelators.

There is, however, a danger in the clinical administration of iron-chelating compounds in that the bacteria may be able to use this chelated iron and improve the uptake of iron leading to the rapid dissemination of bacterial growth. The *in vitro* work of Gomes et al. [91] prompts caution, since some of the chelators examined were associated with increased bacterial proliferation. In a separate study [92], the administration of the iron-chelating agent deferiprone, although effective in iron chelation in thalassaemia and other iron-loaded conditions, was associated with stimulation of the proliferation of *M. avium* in macrophage cell culture. The authors proposed a mechanism whereby chelation of iron from Tf by the drug makes iron more available to the pathogen via the bacterial siderophore carboxymycolactone. Although potential detrimental effects of chelation should be noted, important differences between *M. avium* and *Mtbc* make direct comparisons problematic. Unlike most other mycobacteria, there is a lack of inhibitory growth related to NO (nitric oxide) in *M. avium* and it appears *M. avium* is not as susceptible to oxidative stress damage [93].

Since, for the most part, iron restriction was shown to inhibit *Mtbc* growth in these studies, it is important to determine if excess iron is associated with enhanced growth. Lounis et al. [65] showed that intraperitoneal iron-loading in mice was associated with significantly enhanced multiplication of *Mtbc* in both the spleen and lungs. Again, it was suggested that the iron-withholding capacity of Tf is of crucial importance for the prevention of bacillary multiplication and development of TB, and that the excess iron overcame this capacity leading to enhanced growth. Barclay and Ratledge [94] have taken this one step further and shown that the bacteriostatic effect of the serum (due to the presence of Tf) could be reversed by adding siderophores from *M. avium* and *M. paratuberculosis*. Although these *in vitro* findings can be extended to include *Mtbc* (as the specific siderophores of this bacteria are also able to remove iron from Tf [95]), *in vivo* it may be unlikely that *Mtbc* comes into contact with Tf, because of its intracellular location in the alveolar macrophage [92].

There is some controversy as to the primary *in vivo* iron source for *Mtbc*. In addition to Tf and ferritin, in the lung iron is also bound to lactoferrin and low-molecular-mass chelates. Olakanmi et al. [96] have suggested that lactoferrin is available in a similar or even greater concentration relative to Tf in the lung, and that iron bound to lactoferrin is particularly relevant to pulmonary infections. They have shown that *Mtbc* residing within the phagosomes of human monocyte-derived macrophages was able to obtain iron from these extracellular iron sources and, in fact, obtained 30-fold more iron from lactoferrin than from Tf. It is possible that iron could also be obtained via the contribution from erythrocyte phagocytosis during infection. Conversely, findings from Schaible et al. [97] provide seemingly contradictory evidence to Olakanmi et al. [96] using a β2-microglobulin knockout mouse model (a mouse model with hereditary iron overload providing ample iron for *M. tuberculosis*). The addition of lactoferrin led to decreased extracellular iron availability and was associated with restricted *Mtbc* growth. They also found that NO production that was impaired previously was subsequently corrected with the
addition of lactoferrin. Given these two findings, it is difficult to separate whether it was iron chelation and/or the enhanced NO production associated with lactoferrin that was responsible for the reduction in bacterial growth.

Even though iron-withholding is probably the most important tactic in the initial immune response to Mtb infection, in order to induce pathogenesis bacteria must eventually overcome this obstacle. In response to this challenge imposed by the host, Mtb has developed a variety of circumvention mechanisms to acquire host iron. Mtb grows within the phagocytic vacuoles of the macrophages where the pH is between 6.1 and 6.5 and at which the maximum concentration of Fe^{3+} is only between 1 and 10 ng/ml [92]. Thus Mtb needs a specific mechanism to obtain host iron for its own requirements.

Under conditions of low iron availability, Mtb produce siderophore molecules called mycobactins [98]. Siderophores are high-affinity iron chelators that sequester Fe^{3+} (binding one ion of iron/molecule with a very high-iron affinity: \(K_d\) approx. \(10^{-35}\) mol/l). Mtb produces two iron solubilizing compounds: an intracellular cell-wall associated molecule or mycobactin (in Mtb it is specifically termed mycobactin T) and an extracellular hydrophilic siderophore that is a variation of the hydrophobic mycobactin T molecule termed water-soluble mycobactin T. If this ability to produce siderophores is impaired, as in the case of Mtb H37Rv mutant in which the \(mboB\) gene was deleted, the defective synthesis of mycobactin results in a significant retardation in the ability to grow in the human THP-1 macrophage cell line [99]. Provision of an iron-replete medium reversed the inhibitory effect, demonstrating that iron acquisition from host sources is an essential pre-requisite for growth and pathogenesis.

Iron acquisition from the host in iron-limiting conditions is not the only aspect of iron metabolism which Mtb must be capable of handling. Failure to cope with excess intracellular iron would be lethal to the bacteria due to iron-induced generation of toxic radicals and the resulting cellular damage [92,98,100]. Although mycobactins are produced during iron limitation, a third molecule, bacterioferritin, is synthesized when iron is available in excess. This molecule is related to human ferritin and used to store iron in a form other than mycobactin. Bacterioferritin, unlike mycobactin, is located within the bacterial cytoplasm and can serve to donate iron in a regulated manner, as it is required for bacterial iron-requiring enzymes and proteins.

The varied responses of the bacterial cell to changes in the amount of iron in its environment indicate that there is a complex array of both inductive and repressive effects at the gene level. The co-ordinated response to iron deficiency and the regulation of \(bfrA\), the gene responsible for bacterioferritin, are mediated by a small number of regulators that are dependent on the iron concentration [100].

### Host iron status and TB: susceptibility and clinical outcome

Mtb pathogenicity is a multifactorial process and, although the ability to acquire iron by Mtb is not the sole criterion in this process, it is one of the more important aspects needed for the initiation and establishment of infection. It follows, therefore, that the iron status of the host could be of critical importance. At this time, however, there is a relatively limited amount of quality published work on the actual host iron status antecedent to diagnosis, at the time of diagnosis and during or upon disease resolution. There is also a lack of well-designed research into the potential effects that iron modulation may have on the susceptibility to the development of TB or the duration, severity and mortality associated with this disease in humans. A few studies in humans have provided evidence that a variety of iron parameters are altered in subjects with active TB. Summarizing these studies as a whole is difficult due to the vast differences in study design, population and parameters measured and for the most part an absence of attention to confounders such as HIV status, age, gender, smoking, BMI (body mass index), residence and marital status that have been shown to be important in other studies [101].

### Consequences of elevated host iron in relation to TB

One of the first suggestions that supplemental iron may cause a relapse of TB came from a paper by the French physician Trousseau in 1872 [102]. In this work, he reported subjects recovering from active TB tended to relapse if they received iron-rich dietary supplements (in the form of tonics made from animal blood) in comparison with patients who received no supplements and were somewhat anaemic. Another clue pointing to the potential hazards of iron in relation to the outcome of TB was based on a series of necropsies conducted in Southern Africa between 1925 and 1928 in subjects with normal-to-severe iron overload due to excessive dietary intake [103]. The findings were re-analysed by Gordeuk et al. [104], who revealed that the odds of death were 16.9 times greater in subjects with higher iron stores compared with lower levels (highest splenic iron storage grade compared with negative/trace iron stores combined; 95% CI, 4.8–59.9; adjusting for age, gender, smoking, BMI (body mass index), residence and marital status that have been shown to be important in other studies [101].
of antecedent evidence of iron exposure. One study from Zimbabwe [105] was designed to assess prior dietary iron exposure as a factor in the development of PTB (pulmonary TB). In this region of Africa, beer is brewed in a traditional manner in non-galvanized steel containers leading to an excessively high ferrous iron content. Previous studies had shown that biochemical correlates of iron status were associated with traditional beer consumption in community and hospitalized rural African subjects [106,107]. In the Zimbabwe study [105], a broad approximation of the life-time dietary iron exposure from this source was obtained by interview prior to collection of blood samples. The data revealed that increased dietary iron intake was associated with a 3.5-fold increased odds of developing PTB (95% CI, 1.4–8.9; adjusted for HIV status and liver function) and a 1.3-fold increase in the HR of death (95% CI, 0.4–6.4; adjusted for HIV status, liver function and age). In a separate study published from the same data based on blood taken at 4–6 months post-treatment [108], a 10-fold rise in serum ferritin was associated with a 2.3 increased odds of developing PTB, and a 25-point increase in Tf saturation was associated with a 3.0-fold increased odds ($P = 0.032$ and $P < 0.001$ respectively; adjusted for haemoglobin concentration, erythrocyte sedimentation rate and HIV status). The odds of death were also associated with elevated iron stores (ferritin, 3.6-fold greater odds, $P = 0.027$; and Tf saturation, 2.2-fold greater odds, $P = 0.05$). Although the study designs are associated with certain biases and limitations, the antecedent nature of the dietary iron exposure–outcome relationship is of interest for further follow-up investigations.

Although elevated iron may increase susceptibility to TB, it may also predispose an individual to greater morbidity after TB has developed due to its role in generating ROS. Several studies have demonstrated that oxidative stress (through lipid peroxidation products or diminished antioxidants) is greater in active TB compared with historical TB or healthy controls [109–112]. During pulmonary infection, the lung is exposed to free radicals generated endogenously by active phagocytes, neutrophils and lung alveolar macrophages. Lung injury can be mediated by reactive oxygen, released from active inflammatory cells, which migrate to alveolar spaces in response to alveolar inflammation [112].

The hypothesis that increased iron load predisposes to infection due to increased availability of host iron in vivo is not without controversy. Tf and ferritin possess considerable reserves of iron-binding capacity and even a substantial increase in total body iron burden will not saturate either protein in vivo. It is only when Tf becomes saturated that forms of extracellular iron more immediately available to microorganisms are present in plasma. Until this occurs, Tf saturation is not likely to alter greatly the amount of iron available to the pathogen. There is also the probability that the hypoferraemia of inflammation is only a contributory factor that enhances resistance to infection in concert with other components of the APR such as potentiation of the activity of IFNγ (interferon γ).

Consequences of host iron deficiency in relation to TB

Although evidence has shown that iron excess may predispose to the development of TB, other studies have shown that iron deficiency is also associated with susceptibility to TB. It is likely that the negative impact of insufficient iron on cell-mediated immunity [113], critical for host defence against Mtb, is the key. Using murine models, there was a decreased production of TNFα [114] and IL-12 [115] associated with chelation by DFX or iron deficiency respectively. Unfortunately, the strength of the evidence overall is limited by the fact that conclusions have been extrapolated to human TB from animal studies [114,115], and the determination of iron deficiency or anaemia is often at the time of TB diagnosis [116,117]. This makes it impossible to discount reverse causality due to iron shifts as a result of the inflammatory processes, insufficient dietary intake due to anorexia or malabsorption, or enhanced catabolism due to the effects of the disease.

IRON-REGULATORY GENES

In recent years there has been rapid progress in understanding the molecular mechanisms involved in iron acquisition, transport, storage and recycling in both the human host [118] and its pathogens [1]. The picture emerging is one of great complexity that will take many more years to unravel. For instance, hepcidin, the recently discovered hepatic-derived peptide, has emerged as a strong candidate as the key regulator of intestinal iron absorption and a mediator of some of the infection-induced acute-phase effects on iron redistribution [16–18]. Yet, partly because reliable assays have not yet been widely available, little is known so far about its pathophysiological roles or about possible functional genetic variants.

In selecting the iron-regulatory genes for this review of nutrient–gene interactions two candidates, Hp and NRAMP (SLC11A1), were selected on the basis that: (i) they have common polymorphic variants in humans; (ii) there is strong evidence that the genetic variations translate into distinct biochemical phenotypes with known or expected functional effects; (iii) there is evidence for multiple associations with disease outcomes in humans; and (iv) at least in the case of NRAMP1, there is evidence from animal studies indicating important interactions between the genetic effects and iron status. It is worth noting at this stage that many other iron-regulatory genes that could have potentially interesting effects on host–pathogen interactions [e.g. ferroportin...
physiological role of Hp might possibly lie elsewhere. Animal models [122,123], suggesting that the main function of Hp–haemoglobin complex formation with liver parenchymal cells and macrophages, has led to suggestions that a major function of Hp–haemoglobin complex on liver parenchymal cells is to recycle free haemoglobin thereby reducing iron loss and nephron damage. However, liver clearance and degradation of free haemoglobin thereby reducing renal damage by retarding the passage of free haemoglobin through the glomeruli and diverting it to the liver. This, together with the presence of specific receptors for the Hp–haemoglobin complex on liver parenchymal cells and macrophages, has led to suggestions that a major function of Hp–haemoglobin complex formation is clearance and degradation of free haemoglobin thereby reducing iron loss and nephron damage. However, liver parenchymal cells are capable of taking up free haemoglobin faster than the Hp–haemoglobin complex in animal models [122,123]. Suggesting that the main physiological role of Hp might possibly lie elsewhere. This was confirmed using Hp-knockout mice in whom the capacity and efficiency of free haemoglobin clearance was not impaired [124], suggesting also a level of redundancy in which other acute-phase proteins, such as haemopexin, can substitute for Hp.

Antioxidant effects
Free haemoglobin is a highly toxic molecule; extracellular oxyhaemoglobin spontaneously undergoes an intramolecular oxidation–reduction reaction to generate methaemoglobin and superoxide radical which can cause lipid peroxidation leading to free radical chain reactions [125]. In the presence of H2O2 or lipid peroxide, ferrous or ferric haemoglobin may be oxidized further to ferryl (Fe4+) haemoglobin, a highly toxic compound with a redox potential close to that of the hydroxyl radical. The tight Hp–haemoglobin complex reduces tissue oxidative damage [119]. Lim et al. [124] demonstrated that Hp-knockout mice exhibited a greater degree of oxidative stress (as indicated by higher plasma malondialdehyde and 4-hydroxy-2E-nonenal) and greater renal oxidative DNA damage (as indicated by higher 8-hydroxyguanine concentration) [124,125]. The ability of Hp to protect ascorbic acid from haem-driven degradation is described below in relation to the differential effects of the different genotypes.

The immune response
Hp plays a multifaceted role in immunity and recent studies have suggested that, except under pathological conditions of haemolysis, its immunoregulatory roles may be more important than the conservation of haemoglobin. Suggested immunological roles include: (i) pathogen deprivation of haem iron; (ii) potential effects on inflammation through altered prostaglandin synthesis consequent upon altered levels of arachidonic acid peroxidation due to Hp quenching of the pro–oxidant effects of haem [126]; (iii) action as a serum angiogenic factor influencing tissue repair [127]; (iv) agglutination of T4 antigen by Hp2-2 and Hp2-1 but not by Hp1-1 [128]; (v) modulation of macrophage iron sequestration through interaction of the Hp–haemoglobin complex with the CD163 receptor which occurs with 10-fold greater affinity for the [Hp2-2]–haemoglobin complex than with other Hp variants [129]; and (vi) inhibition of CD22 binding to TNFα-activated endothelial cells, suggesting a likely role in the trafficking of B-lymphocytes [130].

Genetic variation in humans
Hp proteins are composed of α- and β-subunits. In humans, the β-chain lacks genetic variants in all but the very rarest of cases, and the α-chain is polymorphic with two major co-dominant alleles, Hp1 and Hp2, leading to three major phenotypic variants: Hp1-1, Hp2-1 and Hp2-2 (Figure 2).

Functional differences between the major Hp phenotypes

Physical characteristics
Hp concentration varies significantly according to phenotype, with Hp1-1 individuals having the highest concentration (0.57–2.27 g/l), then Hp2-1 (0.44–1.83 g/l), followed by Hp2-2 (0.38–1.50 g/l) [119].

The α1-chains of Hp are physically smaller than the α2-chains making Hp1-1 the smallest Hp protein. As a consequence, Hp1-1 has less restricted access to tissues, whereas Hp2-1, and more so for Hp2-2, are sterically hindered from binding to haemoglobin and have less access to the extravascular space. This functional difference, together with the higher plasma concentration, and a stronger binding constant gives Hp1-1 a higher haemoglobin-binding capacity.
Iron and infection: effects of host iron status and iron-regulatory genes 513

Figure 2  Subunit organization of the major human Hp phenotypes
Hp consists of an N-terminal complement control repeat representing the α-chain and a C-terminal serine-protease-domain-like β-chain. The β-chain has no known catalytic activity. Disulphide bridges between the α- and β-chains link the chains in a (α/β)$_2$ structure. This formation, common to all mammals, is designated Hp 1-1 (α$^1$β)$_2$. Humans also have the multimeric form of Hp having the (α/β)$_2$+n structure (n = 0, 1, 2, etc), where the structural formula for Hp 2-1 is [(α$^1$β)$_2$ + (α$^2$β)$_n$] and for Hp 2-2 is (α$^2$β)$_n$. Due to a duplication of the gene encoding the part of the α-chain involved in inter-α-chain disulphide bridging, each Hp2 α-chain forms disulphide bridges to two other α-chains resulting in a wide range of Hp multimers. Adapted from [119] with permission. © (1996) American Association for Clinical Chemistry.

Immunological characteristics  A lower immune responsiveness has been associated with Hp1-1, whereas Hp2-2 may experience a hyperimmune reaction (reviewed in [131]). Differences in vaccination and immune responses have also been reported, with individuals of the Hp2-2 phenotype generally characterized by a more vigorous immune response to different stimuli than those with the Hp1-1 phenotype. Subjects with Hp2-2 also have higher serum IgM [126] but lower IgA concentrations compared with other phenotypes [132]. Finally, [Hp2-2]–haemoglobin complexes exhibit a 10-fold higher functional affinity for the CD163 receptor on macrophages than [Hp1-1]–haemoglobin [129]. The multivalent exposure of receptor-binding sites in the [Hp2-2]–haemoglobin complexes probably accounts for this difference in affinity. Overall, this may lead to a greater iron loading in the macrophages of individuals with the Hp2-2 phenotype, with hypothesized immunological consequences including the possible promotion of susceptibility to intracellular organisms.

Hp phenotype, iron and ascorbic acid status  Functional differences between Hp phenotypes may influence iron status. Healthy Caucasian males with the Hp2-2 phenotype have been shown to have some degree of iron accumulation in hepatocytes and the mononuclear phagocytic system [133]. Among male subjects, Langlois et al. [133] found that the highest serum iron, ferritin and Tf saturation values were observed for subjects with the Hp2-2 phenotype, whereas serum sTfR (soluble TfR) was significantly lower and Tf concentrations were comparable. The higher serum iron and Tf saturation is suggestive of a relative increase of the iron transport compartment, whereas elevated serum ferritin suggests higher iron stores among Hp2-2 subjects. The lower sTfR suggests a lower cellular expression of membrane
Table 3  Association between haptoglobin phenotype and clinical conditions

For each association, the classification of detrimental or beneficial association was based on the conclusions reported by the original authors. It is possible that the converse may be also true, for example if Hp 2-2 is detrimental, Hp 1-1 may confer a beneficial result for the specified clinical condition. †See text for further discussion and references. ‡Compared with Hp 2-1.

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<th>Phenotype</th>
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<td>Detrimental associations*</td>
<td>HIV-1 infection†</td>
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<td>Reduced life-expectancy in Jordanians‡</td>
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<td>Increased life-expectancy in Japanese</td>
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<td>Hp 1-1</td>
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<td>Beneficial associations</td>
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TfR and, thus, a relative increase in the functional iron compartment. Higher cytosolic l-ferritin concentrations in peripheral blood mononuclear cells suggest more pronounced iron storage in monocytes and macrophages of Hp2-2 subjects, which is similar to subjects with iron overload where only L-ferritin was up-regulated with no differences in H-ferritin concentration. This profile suggests that a less efficient transport of free plasma haemoglobin to the liver associated with Hp2-2 could result in ‘delocalization’ of iron into monocytes/macrophages, leading to enhanced iron storage.

However the study by Langlois et al. [133] failed to find any associations with markers of iron status in females. Other groups [134,135] also failed to find an association between Hp phenotype and markers of iron status (serum ferritin and Tf saturation) in healthy male and female subjects. Although differences in the reference Hp concentrations between Caucasian and Zimbabwean subjects have been reported [136], the evidence again suggests a lack of association between Hp phenotype and iron status (serum iron, total iron-binding capacity, Tf saturation and ferritin concentrations) among healthy Zimbabwean male and female subjects [137]. Differences in iron markers in HIV+ patients are summarized below.

If Hp2-2 has a lower haemoglobin-binding capacity (due to the different molecular masses and resulting accessibility to interstitial fluid) and if there is a greater degree of iron accumulation (which could affect iron-driven ROS production), then oxidative stress may be associated with the Hp2 allele. In a study by Langlois et al. [138] healthy Caucasian subjects with the Hp2-2 phenotype had lower serum concentrations of vitamin C, and in vitro experiments showed a lower stability of l-ascorbic acid, due to increased haemoglobin-driven oxidation of l-ascorbic acid to l-dehydroascorbic acid.

Given the conflicting evidence regarding the association between Hp phenotype and iron status, a firm conclusion in healthy subjects is not yet possible. This does not, however, rule out the possibility of clinically significant differences in pathological conditions.

Hp and disease susceptibility

Clinical conditions associated with Hp phenotypes

Table 3 summarizes the wide range of clinical conditions that have been associated with the major Hp phenotypes.
The existing evidence suggests that each phenotype may present an advantage in certain clinical conditions, thus providing a rationale for the continued presence of both the \( Hp^1 \) and \( Hp^2 \) alleles within the population. It was initially speculated that the relatively rapid penetration of the \( Hp^2 \) allele throughout the world, from what is thought to be a single mutation event in Southern India some 2 million years ago, was driven by a protective advantage against malaria [157]; however, its penetration into non-malarious latitudes suggests that there must be an alternate or additional driver.

**Hp and HIV infection**

The risk of initial infection with HIV does not seem to be affected by Hp phenotype, as there is no evidence that allele frequencies differ between either African [158] or Caucasian [159,160] HIV-1 seropositive cases and healthy controls. However, there is strong evidence that the outcome after HIV-1 seroconversion may be affected. In a cohort study of Belgian subjects (male and female, antiretroviral naïve, prior to the introduction of protease inhibitors), those with the Hp2-2 phenotype had a significantly higher mortality (Hp2-2 compared with Hp1-1/2-1: median survival, 7.3 compared with 11.0 years) and corresponding mortality risk ratio of 1.78 (95% CI, 1.25–2.54; after adjustment for age, gender) [160]. Although ideally the baseline degree of immunosuppression should also have been adjusted for in this model, they did provide evidence that the plasma HIV-1 RNA levels at baseline were significantly higher among subjects with the Hp2-2 phenotype (Hp2-2, 5.26 ± 0.82 log\(_{10}\) RNA copies/ml; Hp1-1, 3.75 ± 1.01 log\(_{10}\) RNA copies/ml; Hp2-1, 4.64 ± 0.73 log\(_{10}\) RNA copies/ml; \( P = 0.03 \); values are means ± S.D.). After the introduction of protease inhibitors and follow-up for 1 year, subjects with Hp2-2 also exhibited the highest viral load increase (Hp2-2 compared with Hp1-1/2-1, 0.52 ± 0.38 compared with 0.28 ± 0.30 log\(_{10}\) RNA copies/ml respectively; \( P = 0.003 \)). This is a clinically significant finding as viral load is a strong predictor of disease progression and/or mortality in HIV-1 [161]. In the Belgian study [160], peripheral CD4+ cell count decline (over a median follow-up time of 48 months) did not differ significantly but, in Ghana [162], a cross-sectional study demonstrated that baseline CD4+ counts were significantly lower in subjects with Hp2-2 phenotype compared with either Hp2-1 or Hp1-1. The limited evidence provided by these two studies does not allow firm conclusions concerning the role of Hp phenotype and immunosuppression in HIV-1 infection.

Delanghe et al. [160] observed that a greater degree of iron accumulation was associated with the Hp2-2 phenotype in male and female HIV-positive subjects and controls (significantly higher serum iron, Tf saturation and ferritin, but not Tf concentrations). The authors speculate that it is the elevated iron status, combined with the lower Hp concentration and the reduction in the antioxidant ascorbic acid, that would increase the likelihood of oxidative stress leading to enhanced viral replication. This could explain the negative impact on HIV-associated mortality. As with Delanghe et al. [160], a recent case-control study (Italian male and female HIV-1 seropositive subjects and healthy controls, antiretroviral naïve and hepatitis B virus and hepatitis C negative) also reported that Hp2-2 phenotype was associated with a more advanced disease stage, higher serum ferritin and lower citrate concentrations than either Hp2-1 or Hp1-1 phenotypes [159]. Unfortunately, the actual data to support this claim were not presented, as the observation was only presented in the discussion section of the study report.

**Hp, susceptibility to TB and clinical progression**

The number of functional, immunological and biochemical differences between Hp phenotypes provides a strong indication that Hp phenotype could influence susceptibility to the development of TB, the clinical course during active infection and the possibility of recurrent TB. Specifically, the following hypothesized mechanisms could yield phenotypic differences.

(i) Oxidants generated by activated macrophages are involved in acute tubular necrosis. Haemoglobin-binding capacity may reduce the likelihood of developing this complication; thus, Hp1-1 would be the most protective against an unfavourable clinical outcome.

(ii) A greater susceptibility and poorer prognosis may be related to the possibility of a greater degree of iron accumulation associated with Hp2-2. Elevated macrophage iron in Hp2-2 subjects could diminish host immune responses and favour bacterial iron acquisition. Elevated iron elsewhere could propagate iron-driven oxidative stress and the clinical consequences associated with this process.

(iii) The balance between the production of Th1 and Th2 cytokines is an important determinant of host resistance to TB. Disruption of this balance in favour of Th2 could affect the ability of the host to express a protective immune response against \( Mtb \). Given that Hp2-2 is associated with the lowest concentration of serum Hp and that Hp may favour a Th1 (cell-mediated response), Hp2-2 may be associated with a negative outcome.

At this time, however, there are very few published human studies which support or refute these possible associations and mechanisms. A number of studies in the Russian medical literature have been published over the years examining the role of Hp on TB (for example, [163–166]). Overall, it appears that Hp2-2 was associated with the following: greatest likelihood of developing TB; a poorer outcome after infection (slower resolution of
infiltrative changes, greater number and longer recovery from chemotherapy-associated side-effects, greater occurrence of endobronchitis and bronchial obstruction and advanced dissemination); and greater likelihood of disease recurrence. Hp1-1 seemed to exist as an intermediate risk factor, whereas Hp2-1 was associated with significantly fewer cases of TB and earlier resolution of active disease (abacillation of sputum, cavity closure and enhanced immune response). Although these studies are difficult to critique in detail, the consistency between them provides a rationale for further investigations.

In contrast with the Russian studies [166], among Indonesian subjects, Grange et al. [167] found no differences in the Hp phenotype distribution between PTB subjects (n = 97) and healthy controls (n = 126), nor did they find an association between phenotype and lymphocyte count, dermal reactivity to tuberculin or the degree of suppression. Likewise, among Zimbabweans, no significant differences were observed in Hp phenotype distribution between clinical PTB cases (n = 98) and healthy controls (n = 98) [168]. They did, however, observe that after a median follow-up of 6 months, the odds of dying among those with the Hp2-2 phenotype were 6.1 times greater than those with Hp1-1 (95% CI, 1.04–35.1; adjusting for age, gender and haemoglobin concentration). Further adjustment for HIV-seropositivity in the same regression model resulted in a slight decrease in the odds ratio to 5.9 (95% CI, 1.02–34.1). These results suggest that, in this population, Hp phenotype was not associated with initial establishment of PTB infection, but may affect mortality after treatment initiation.

**NRAMP1**

NRAMP1 is a divalent cation transporter in the phagosomal membrane of macrophages that influences resistance to intracellular pathogens [169,170]. The evidence leading to this conclusion started to emerge over 30 years ago with a series of elegant studies conducted in mice genetically resistant (BcgG169, dominant) or susceptible (BcgD169, recessive) to *M. bovis* [BCG (bacillus Calmette-Guérin)]. This represented the discovery of the first non-HLA gene associated with early growth of mycobacteria (reviewed in [171]). Differences in susceptibility to infection in mice also included a wide variety of intracellular pathogens: *M. lepraemurium, M. intracellulare, Toxoplasma gondii, Leishmania donovani, L. infantum* and *Salmonella typhimurium* [172–175]. Using the previous gene nomenclature these were first shown for *Salmonella* (Ity) and *Leishmania* (Lsh).

Studies of mice with the BcgG and BcgD genes revealed that a naturally occurring glycine > aspartate mutation at codon 169 in transmembrane 4 of NRAMP1 results in a functional null mutation affecting protein stability and/or targeting, ultimately resulting in the absence of mature protein in BcgG mice.

**NRAMP1 genetic and protein structure**

Immunocytochemical studies show that NRAMP1 is exclusively expressed in late endosomal and lysosomal membrane fractions of macrophages and, upon phagocytosis, NRAMP1 translocates to the phagosomal membranes [176,177]. In humans, NRAMP1 gene expression has been detected in spleen and liver and more strongly in the lung; however, expression is most abundant in polymorphonuclear cells [178], suggesting NRAMP1 polymorphisms could have been driven by the evolutionary pressure of *Mt b*.

NRAMP1 polymorphisms are distributed along the entire NRAMP1 genomic sequence and a complex LD (linkage disequilibrium) pattern exists within and around the NRAMP1 locus [179,180]. The SLC1 polymorphism is a complex dinucleotide repeat that is functional by influencing NRAMP1 expression. Searle and Blackwell [181] reported that allele 3 drives higher expression relative to the other alleles (5–8-fold greater expression), and the magnitude of the differences varies according to levels of external stimuli by LPS (lipopolysaccharide). Such effects have not yet been validated on endogenous genes.

**Physiological functions of NRAMP1**

The function of the human NRAMP1 protein has not yet been fully elucidated, but evidence for its role has been inferred from animal studies. Using the Belgrade (*b*) rat or *mk* mouse [which have a mutated NRAMP2 form (Gly185Arg), leading to defective iron absorption in the intestine and tissues with subsequent development of microcytic anaemia], it was hypothesized that NRAMP2, and its parologue NRAMP1, play a role in iron transport. Jabado et al. [182] provided direct evidence of this when they observed that iron chelation (DFX and salicyladehyde isocontinoyl hydrazone) produced similar effects on intracellular bacterial growth as seen in NRAMP1-knockout mice. Experimental induction of murine murine NRAMP1 by treatment of macrophages with bacterial endotoxins and cytokines [GM-CSF (granulocyte/macrophage colony-stimulating factor), IFNγ, LPS and IL-1] demonstrated that the NRAMP1 protein also exhibits pleiotropic effects on macrophage activation, including regulation of chemokines, increased expression of iNOS (inducible NO synthase), up-regulation of MHC II molecules, increased production of the pro-inflammatory cytokines IL1-β and TNFα, increased NO release, and production of ROS and reactive nitrogen intermediates involved in the oxidative burst (reviewed in [183]). Overall, these effects appear to play a critical role in early innate macrophage responses to intracellular infection in mice.

It has been demonstrated *in vitro* that NRAMP1 can act as a proton/divalent cation transporter [184]. The
first evidence that NRAMP1 may function as a divalent cation transporter emerged from studies using its yeast homologue SMF1, which demonstrated that SMF1 could transport Mn^{2+}. Later Zwilling et al. [185] revealed a clue supporting a role for iron transport when they reported lower cellular iron levels in macrophages with the NRAMP1 wild-type allele compared with those possessing the mutant NRAMP1. Additionally, they demonstrated that the addition of iron influences mRNA expression post-transcriptionally. The direction of cation transport depends on the pH and cation concentration, and further work by this group [186] is not inconsistent with the hypothesis that NRAMP1 can function to transport iron into the bacterium-containing phagosome where it serves as a catalyst to the Haber–Weiss reaction.

Two schools of thought have evolved as to the true biochemical nature of NRAMP1 and how it might provide protection from intra-phagosomal colonization by bacteria (reviewed in [187]).

The first school focuses on the hypothesis that NRAMP1 functions to increase intraphagosomal Fe^{2+} in order to provide a catalyst for the Haber–Weiss/Fenton reaction, thereby generating highly toxic hydroxyl radicals for host bactericidal activity [188–190].

The alternative theory is that NRAMP1 functions to deprive the intra-phagosomal bacterium of iron and other divalent cations [182,187,191,192]. Divalent metals such as Zn^{2+}, Mn^{2+} and Fe^{2+} are all substrates for NRAMP proteins and they are also essential micronutrients for the pathogen. These metals act as cofactors in many enzymatic reactions, including bacterial antioxidant defence; therefore their depletion from the phagosome by NRAMP1 could have a simple and broad-spectrum bacteriostatic effect. Depletion might also enhance the bactericidal activity of host macrophages by rendering the pathogen more sensitive to killing by oxygen radicals.

Forbes and Gros [191] suggest that, although appealing, a model whereby iron is pumped into the phagosome has potential problems. First, pumping iron into the phagosome would go against the function established for NRAMP2 with respect to the topological orientation of the two proteins in the membrane. They suggest it is unlikely that proteins as similar as NRAMP1 and NRAMP2 (64% shared amino acid sequences, 78% overall similarity and 86% similarity in the transmembrane domains) [178] would have different membrane orientations or function by different mechanisms. Secondly, they studied this paradox using a method which monitors fluxes of divalent metals in individual phagosomes in vivo and in real time. From their results, they conclude that NRAMP1, like NRAMP2, transports divalent metals down a proton gradient, acting like a phagosomal pump [191]. This view is supported by in vitro evidence using macrophage cell lines which showed a reduced intracellular iron accumulation and increased iron efflux in cells transfected with the resistant compared with the susceptible alleles [193].

**MntH: a mycobacterial NRAMP1 orthologue**

Given the diversity of species with NRAMP1 genes [194,195], it is not surprising that the genomic sequencing of *Mtb* revealed a similar gene, *mntH* (Rv0924c) [196], with comparative analyses indicating a 26–29% sequence identity and overall similarity of 40–45%. In vivo, *mntH* mRNA expression was stimulated by low iron concentrations, suggesting that *mntH* in *Mtb* may act as a divalent metal import system within the iron-restricted environment of the host phagosome [197]. This suggests a model for NRAMP1 function in which both the mammalian and bacterial NRAMP1 proteins act on the same group of substrates within the intraphagosomal space, both in a pH-dependent manner, but in opposite directions, and hence in competition. Although this may be the case, in a previous study [198] inactivation of *mntH* using a knockout mutant of *Mtb* H37Rv did not affect virulence in bone-derived macrophages or in vivo in a murine model of TB. NRAMP1 proteins may be important in human TB, but at the same time there may be sufficient redundancy in the cation acquisition systems of *Mtb* in order to overcome the loss of *mntH*.

**Clinical significance of NRAMP1 polymorphisms**

Selective pressure for genetic polymorphisms exists when a disease has a significant effect on morbidity and mortality before reproductive age, and this effect has been exerted over a long period of time. If different alleles remain in the population over time, it suggests that there are balancing selective forces maintaining their frequencies. This may occur when polymorphisms are protective in some diseases and promote others. The fact that a number of different genetic polymorphisms exist in NRAMP1, together with pronounced geographic and ethnic variation, suggests that they may be sustained by a variety of disease pressures (reviewed in [199]).

In humans, NRAMP1 polymorphic variants or haplotypes have been associated with susceptibility or resistance to multiple infections and conditions as listed below (reviewed in Blackwell [200], except where individual references are listed): (i) viral infection (HIV-1 in Columbians); (ii) bacterial infections (TB, leprosy in Vietnamese and severe clinical meningococcal meningitis in Africans); (iii) protozoal infections (visceral leishmaniasis in Brazilians and post-kala-azar leishmaniasis in Africans); (iv) atopic diseases (food allergy, atopic dermatitis, allergic asthma and rhinoconjunctivitis in Nordic ethnic groups [201]); (v) autoimmune
diseases (multiple sclerosis in South African Caucasians, sarcoidosis in African Americans, rheumatoid arthritis in Canadian Caucasians and Koreans, and juvenile rheumatoid arthritis in Latvians [202]); (vi) Type I diabetes in the UK [203]; and (vii) inflammatory bowel disease in Japanese.

NRAMP1 and susceptibility to HIV infection

Despite the hypothesized role that NRAMP1 may play in regulating the macrophage microenvironment and thus the potential effect it would have on macrophage functioning in the host immune response, susceptibility to HIV-1 infection and NRAMP1 polymorphisms has only been studied in a single report [204]. In this population-based case-control study from Columbia, four NRAMP1 genotypes (SLC1–4) were each associated with a significantly altered risk of HIV infection, whereas two others (SLC6a and SLC6b) did not show statistically significant associations. Since the SLC1, SLC2 and SLC3 markers are in LD with each other, independent effects cannot be attributed to any single polymorphism. However, they are not in LD with SLC4, suggesting independent effects between these two regions. Multiple regression analyses of the combined effect of these four polymorphisms did not demonstrate additional combined effects.

In this study [204], alternative explanations that may have produced spurious associations, such as the effect of population substructure and the presence of the chemokine receptor 5 gene (CCR5)-Δ32-bp deletion allele (known to alter the risk of HIV infection) were ruled out. Nonetheless, since NRAMP1 is located in the gene-rich human chromosome 2q35 region, it is possible that alleles of a second gene in tight LD with NRAMP1 could be the underlying cause of these non-random associations with IL8R (gene encoding IL-8 receptor) being a likely candidate, since the expression of IL8R is significantly reduced in HIV-seropositive subjects.

NRAMP1 and susceptibility to TB

Numerous studies have provided evidence that host genetic factors are important determinants of susceptibility to TB and one of the best characterized thus far is that of NRAMP1. The first reported study to examine this was a case-control study from hospital-based recruitment in The Gambia [205]. After adjustment for ethnicity and other pertinent factors, significant associations were observed with four allelic variants three of which were in strong LD. Since that publication, various authors have reported associations between NRAMP1 polymorphisms and TB in a number of genetically distinct populations (summarized in Supplemental Table 1 at http://www.clinsci.org/cs/110/cs1100503add.htm). It is evident, however, that there is a lack of consistency between studies. Genome-wide scans in Gambian and South African populations have also failed to identify NRAMP1 as a major candidate locus [74]. Thus, in spite of the strong theoretical basis on which to predict that NRAMP variations could represent susceptibility factors and encouraging initial reports, the evidence is thus far still weak.

NUTRIENT–GENE INTERACTIONS AND THE CONCEPT OF EFFECT CONCENTRATION

Failure to replicate association studies has emerged as a consistent theme in genetic research and has been attributed largely to a combination of inadequate sample sizes that yield chance observations and positive reporting bias [206]. Another possible explanation is that, as would be predicted, genes express different functional effects in different environmental conditions. In the case of iron-related genes, their effects on disease risk might depend on the underlying iron status of the population. For example, if NRAMP1 protects against intracellular organisms by depleting iron from the macrophage, then the effect may be most potent in an already iron-deficient subject and any effects could be nullified by the addition of iron. Thus the iron status of the population under study could affect the likelihood of detecting associations. Furthermore, even within certain populations, there may be a wide heterogeneity of effects across the range of iron status in which the ‘true’ function of a gene is only apparent against the conditions of its evolutionary selection (e.g. persistent iron deficiency). This can be termed ‘effect concentration’ and is an important concept to bear in mind when attempting to study nutrient–gene interactions.

Unless factored into the analysis, such gene–environment interactions will obscure the relationship. This represents a major challenge for the future, especially as hypothetical power calculations indicate the need for sample sizes in the tens of thousands when investigating even a two-way interaction term.

ANIMAL EVIDENCE FOR GENE–NUTRIENT INTERACTIONS

Two animal studies have examined the effect of supplemental iron on the ability of NRAMP1 to protect against M. avium. Research both in vitro in macrophage culture and in vivo in mice has shown that the growth rate of M. avium is highly dependent on the amount of iron available and that the protective effect of Bcg' disappears in the presence of high iron levels (reviewed in [93]) (Figure 3). Zwilling et al. [185] reported that the addition of extracellular iron inhibited intracellular M. avium replication in macrophages from Bcg' mice, whereas it
stimulated growth in Bcg' macrophages, and that these effects occurred over a very narrow concentration range (0.005–0.5 µmol/l). Although intriguing, these results should be extrapolated to humans with great caution due to the inherent differences between murine M. avium infection and human Mtb.

CONCLUSIONS

Although there remains much to be learnt, the evidence presented above strongly suggests that iron plays a pivotal role in host–pathogen interactions and that this is likely to be modulated by genetic variations in both the host and the pathogen. Host–pathogen battles are fought over widely varying timescales. In the short term, both host and pathogen invoke a range of attack and defence mechanisms involving iron within the first minutes, hours and days of exposure to infection. If the initial infection gains a foothold, then in the medium term competition for iron continues as the host tries to survive, and medical intervention with supplemental iron can exacerbate infection and increase mortality. In the very long term, evolutionary selection alters the genome of both the host and its pathogens by selecting for iron-related traits. The examples discussed above in terms of Hp and NRAMP1 provide only a glimpse of what remains to be discovered and emphasizes the importance of research into gene–environment interactions (among which nutrients are among the most potent of the environmental exposures that need to be examined).

Studying these interactions will be extremely challenging. The study of interactions requires very large sample sizes even if the effects are quite strong. This problem is compounded by the fact that, by definition, the evolutionary battle between host and pathogen must be finely poised; if this were not the case, either the host or the pathogen would be extinct. The challenge for clinical medicine is therefore to untangle the circumstances when these subtle effects can have important implications for therapeutic practice. The widespread distribution of iron in developing countries where pathogen loads remain high is one example where there is an urgent need to understand the underlying mechanisms of iron and infection in order to refine supplementation and therapeutic regimes.

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