Iron deficiency and *NRAMP1* polymorphisms (INT4, D543N and 3’UTR) do not contribute to severity of anaemia in tuberculosis in the Indonesian population

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*(Received 3 November 2006 – Revised 14 March 2007 – Accepted 14 March 2007)*

Fe-deficiency anaemia is the most common cause of anaemia in developing countries. In these settings, many chronic infections, including tuberculosis (TB), are highly prevalent. Fe is an essential nutrient for both host and mycobacteria that play a pivotal role in host immunity and mycobacterial growth. A case–control study was performed in a TB-endemic region in Jakarta, Indonesia, among 378 pulmonary TB patients and 436 healthy controls from the same neighbourhood with the same socio-economic status. In a number of these subjects the Fe status could be explored. The distribution of three polymorphisms in the natural resistance-associated macrophage protein gene (*NRAMP1*) including INT4, D543N and 3’UTR was examined for a possible association with susceptibility to TB. Anaemia (corrected for sex) was present in 63.2% of active TB compared with 6.8% of controls, with female patients more often affected. Anaemia was more pronounced in advanced TB as diagnosed by chest radiography. Lower Hb concentrations in TB patients were accompanied by lower plasma Fe concentrations, lower Fe-binding capacity and higher plasma ferritin. After successful TB therapy, Fe parameters improved towards control values and Hb levels normalised, even without Fe supplementation. *NRAMP1* gene polymorphisms were not associated with TB susceptibility, TB severity or anaemia. In conclusion, most active TB patients had anaemia, which was probably due to inflammation and not to Fe deficiency since TB treatment without Fe supplementation was sufficient to restore Hb concentration.

**Tuberculosis: C-reactive protein: Anaemia: Iron: NRAMP1**

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*MTB*), is a global health threat, with 8 million active new cases and 2–3 million deaths annually. The majority of TB cases reside in developing countries, among others in Indonesia, which harbours more than 10% of TB cases worldwide¹. *MTB* is an intracellular pathogen that targets host phagocytes, and consequently an effective host defence is required to coordinate cellular immune response². Following phagocytosis, *MTB* lives within phagosomes of host macrophages and competes with the host to acquire Fe in order to survive and replicate³. For the host, Fe is an essential component of Hb, as Fe binds and transports O₂. Fe is also needed for electron transport, DNA synthesis and immune function, for example, for the formation of oxygen radicals⁴.

It remains unclear how *MTB* accumulates Fe in macrophages. An excess of Fe supply will result in *MTB* growth, and Fe overload is a known risk factor for infections, as this may worsen the disease. Fe overload, for example from dietary Fe, causes individuals to be more susceptible to TB⁵. Interestingly, the *MTB* growth within macrophages from individuals with hereditary haemochromatosis, a genetic disease which leads to Fe overload, is reduced⁶. This can be explained by the fact that, even in severe Fe overload,

Abbreviations: ACD, anaemia of chronic disease; CRP, C-reactive protein; CXR, chest X-ray radiography; ESR, erythrocyte sedimentation rate; MCH, mean corpuscular Hb; MCV, mean corpuscular volume; *MTB*, *Mycobacterium tuberculosis*; NRAMP1, natural resistance-associated macrophage protein; TB, tuberculosis.

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macrophages in hereditary haemochromatosis provide an Fe
deficient environment due to increased export of Fe from the
labile Fe pool to plasma by the export protein ferroportin7,8.

On the other hand, Fe deficiency can increase susceptibility
to various infectious diseases, since macrophages require Fe to
function well7. Even mild Fe deficiency causes a significant
impairment in the immune status and reduces the capacity
to control infections. Fe-deficiency anaemia is the most
common cause of nutritional deficiency anaemia in developing
countries, affecting mostly children and pregnant and lactating
women9. Therefore, Fe supplementation is often prescribed in
developing countries10.

Several susceptibility-associated genetic polymorphisms
have been proposed to explain differential susceptibility to
TB. Various studies have reported that subtle variations in
the natural resistance-associated macrophage protein gene
(NRAMP1) result in a higher risk for having TB11–13. NRAMP1
is a metal transporter protein localised in late endo-
somal and lysosomal compartments, and probably plays an
important role in transferring diferric Fe across the phagosome membrane14.15.

In the present study we conducted a case–control study in
Indonesia, a country with a high prevalence of both TB and
Fe-deficiency anaemia. Fe status of pulmonary TB patients and
controls was explored to determine the prevalence of
anaemia with or without Fe deficiency in active TB patients.
Also, the effect of TB infection on Fe status indicators during
the course of TB therapy was investigated, as inflammation
affects many indicators of Fe status10. Distribution of
NRAMP1 alleles and genotypes in Indonesia has not been
reported before. In the present study, three commonly inves-
tigated polymorphisms in the NRAMP1 gene, INT4, D543N
and 3’UTR, were examined to explore whether such poly-
morphisms are associated with susceptibility to TB or TB
severity.

Materials and methods

Subject recruitment

After written informed consent was obtained from all subjects,
494 newly detected sputum smear-positive pulmonary TB
patients aged over 15 years were recruited in a poor setting
area at an out-patient TB control clinic, Perkumpulan Pember-
antasan Tuberkulosis Indonesia (PPTI), in Jakarta from Janu-
ary 2002 until December 2005. This case–control study was
part of a larger TB study in Indonesia. The study design
was approved by the Medical Faculty University of Indonesia
and the Eijkman Institute Jakarta ethical committees. Pulmonary
TB was diagnosed based on the clinical presentation, chest
X-ray radiography (CXR), and confirmed by two consecutive
acid-fast bacilli-positive sputa. All patients were provided
with free anti-TB therapy according to the national TB pro-
gramme (2HRZE/4H3R3). Patients with seropositive HIV (n
7; 1.4 %), diabetes mellitus (n 96; 19.4 %), CHD (n 3;
0.6 %) or incomplete data (n 10; 2.0 %) were excluded from
the statistical analyses. For analysis after therapy, only
patients with complete medical records after TB therapy (n 153)
were included.

In the same period, 519 healthy controls from the
neighbourhood where the cases lived, with the same
socio-economic status, were randomly selected and matched
for sex and age (± 10 %). Controls were interviewed using
the same standardised questionnaire and underwent the
same physical and blood examination and CXR as cases.
Control subjects with CXR suggestive of TB (n 17; 3.3 %)
or history of prior anti-TB treatment (n 7; 1.4 %), diabetes
mellitus (n 25; 4.8 %) and incomplete data (n 34; 6.5 %)
were excluded. Although not all control subjects were
tested for HIV status, since informed consent for HIV test-
ing in the control group could only be obtained later in the
study, HIV seropositivity was only found in two of 115
(1.7 %) tested controls. Indonesia has a low HIV prevalence
in the general population, which was similar to the number
found in TB patients and in accordance with earlier reports
for Indonesia1.

Blood samples were obtained by venepuncture. Full blood
counts were performed routinely in the clinic for all patients
before therapy and all controls using an automated blood ana-
lyser (Cell-Dyn 3200, Abbott Laboratories, Abbott Park, IL,
USA). Haematology data could be obtained only in sixty-
five of 153 patients after therapy since full blood counts are
not routinely performed in the clinical setting.

Plasma from heparinised blood was collected and stored at
−80°C for further analysis. Fe status indicators including
plasma Fe, plasma Fe-binding capacity, Fe saturation and
plasma ferritin were measured from patients for whom haema-
tology data were available (n 65) and in a set of randomised
controls (n 76). Total plasma Fe was measured using an ascor-
bate/FerroZine colorimetric method (Abbott Laboratories,
Abbott Park, IL, USA). The plasma ferritin was measured by
a solid-phase, two-site chemiluminescent immunometric
assay (Immulite 2000; Diagnostic Product Corporation, Los
Angeles, CA, USA). Fe status indicators could, however,
only be measured in the plasma of thirty-three patients after
therapy due to limited plasma availability. Erythrocyte sedi-
tementation rate (ESR) and/or C-reactive protein (CRP) examina-
tion were measured as indicators of the inflammatory
response.

Genotyping of NRAMP1 single nucleotide polymorphisms

Genomic DNA was isolated from EDTA blood of patients
and controls. Two single nucleotide polymorphisms in the
gene NRAMP1, D543N (1703G > A in exon 15 leading to
an aspartate to asparagine substitution at codon 543) and
INT4 (a single nucleotide change in intron 4; 469 + 14G > C), were analysed11. Multiplex assays were
designed using Assay Design software (Sequenom Inc.,
San Diego, CA, USA). Genotyping was performed using the
MassArray platform according to the manufacturer’s
protocols (Sequenom Inc.). In brief, after PCR on 2.5 ng
DNA a primer extension reaction was performed to intro-
duce mass differences between alleles and, after removing
salts by adding a resin, about 15 nl of the product was
spotted onto a target chip with 384 patches containing
matrix. Mass differences were detected using a Bruker Auto-
flex matrix-assisted laser desorption ionisation time-of-flight
(MALDI-TOF) mass spectrometer (Sequenom Inc., San
Diego, CA, USA) and genotypes were assigned real-time
using Typer 3.1 software (Sequenom Inc.). As quality
control, 10 % of samples were genotyped in duplicate and
no inconsistencies were observed. Primer sequences are available upon request.

Genotyping using fragment length analyses

PCR for a TGTG deletion polymorphism in the 3’ untranslated region (1729 + 55del4), denoted as 3’UTR, were performed using 100 ng genomic DNA, 200 μM of each dNTP, 10 pmol of each primer, 50 mM-KCl, 10 mM-tri(hydroxymethyl)-aminomethane-HCl (pH 9·0), 0·1% Triton X-100, 1·5 mM-MgCl2 and 0·5 U Taq DNA polymerase (Biolabs, Beverly, MA, USA) in a total volume of 25 μl. Forward primers were 5’-labelled with tetrachloro-6-carboxy-fluorescein (TET); primer sequences and cycle conditions are available on request.

PCR products and a 400 HD-ROX size standard (Applied Biosystems, Foster City, CA, USA) in HiDi formamide were run on an ABI Prism 3700 DNA Analyzer (Applied Biosystems), and results were analysed using GeneScan Analysis and Genotyper software (Applied Biosystems). Several homozygous alleles were sequenced to verify allele lengths.

Statistical analysis

Data from the questionnaires, physical examinations, laboratory analyses and genotypings were analysed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Data were checked for normality using the Kolmogorov–Smirnov test. Independent and paired t tests were used to compare means. Ferritin concentrations were transformed to natural logarithms to obtain normality. Analysis of covariance was used to compare indicators of Fe status.

The Hardy–Weinberg equilibrium of each polymorphism was checked using the program HWE17. The program CONTING was used to calculate χ² and associated values for a contingency table17. All statistical analyses were two-sided and P values <0·05 were considered as statistically significant.

Results

A total number of 378 newly detected sputum-positive pulmonary TB patients (median age 29 (range 15–67) years) and 436 community healthy controls (median age 33 (range 15–70) years) were included. Clinical characteristics of all included TB patients and controls are presented in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics of pulmonary tuberculosis (TB) patients before and after TB therapy compared with healthy community controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mean values and standard deviations)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pulmonary TB</th>
<th>Before therapy (n 378)</th>
<th>After therapy (n 153)</th>
<th>Controls (n 436)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Males (n)</td>
<td>225</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td>Females (n)</td>
<td>153</td>
<td>71</td>
<td>191</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>17-6</td>
<td>2·7</td>
<td>19-7</td>
</tr>
<tr>
<td>Inflammatory indicators</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>84·0</td>
<td>32·8</td>
<td>18·2</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>62-6†</td>
<td>43·5</td>
<td>7·7</td>
</tr>
<tr>
<td>Haematological indicators</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaemia (n)§</td>
<td>239</td>
<td>63·2</td>
<td>5</td>
</tr>
<tr>
<td>Anaemia (%)§</td>
<td>63·2</td>
<td>7·6</td>
<td></td>
</tr>
<tr>
<td>Hb (g/l)§</td>
<td>119</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>76·9</td>
<td>8·1</td>
<td>139</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>25·9</td>
<td>4·4</td>
<td>28·1</td>
</tr>
<tr>
<td>Fe status indicators</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin (μmol/l)¶</td>
<td>313-7</td>
<td>289·7</td>
<td>71·9**</td>
</tr>
<tr>
<td>Serum Fe (μmol/l)</td>
<td>8-2‖</td>
<td>3·5</td>
<td>16·0**</td>
</tr>
<tr>
<td>Fe saturation (%)</td>
<td>19-6‖</td>
<td>13·4</td>
<td>32·3**</td>
</tr>
<tr>
<td>Fe-binding capacity (μmol/l)</td>
<td>42-8‖</td>
<td>10·1</td>
<td>52·4**</td>
</tr>
</tbody>
</table>

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; MCV, mean corpuscular volume; MCH, mean corpuscular Hb.

*All values before TB therapy were significantly different compared with controls (t test). All values before TB therapy were also increased or decreased significantly (P<0·05) at the end of the therapy (paired t test, tested only in individuals with two time points).
† Data were collected from 240 individuals.
‡ Data were collected from 295 individuals.
§ Criteria for anaemia: males Hb < 130, females Hb < 120 g/l.
‖ Data were collected from sixty-five individuals.
¶ Male and female values were taken together.
** Data were collected from thirty-three individuals.
†† Data were collected from seventy-six individuals.
Anemia in active tuberculosis and its association with clinical presentation

Active TB was a strong predictor of lower Hb concentrations. In the patients, Hb concentrations were significantly correlated to CRP concentrations (P=0.013), but not to ferritin concentration (P>0.2; analysis of covariance controlling for age and sex). Anaemia was found in 239 active TB patients (63.2 %) compared with only thirty controls (6.8 %). Females were more often affected by anaemia than males, both among TB patients (74.5 v. 55.6 %) and among controls (10.9 v. 3.6 %). At the end of successful TB therapy, anaemia had been corrected without any Fe therapy or dietary supplementation in almost all patients. Hb concentrations were 20 g/l higher after treatment (paired t test; P<0.001). The increase of Hb concentrations between recruitment and end of TB treatment was associated with a decrease of the inflammatory indicators such as ESR and CRP. Also mean corpuscular volume (MCV) of erythrocytes and mean corpuscular Hb (MCH) levels increased (Table 1). Only five patients (7.6 % of sixty-five measured post-therapy) remained anaemic but their Hb concentrations increased significantly.

TB patients had coughing (98 %) as their main complaint. As TB is a chronic disease, the duration of the main complaint before TB patients presented themselves in the clinic might be of importance. Anaemia was more prevalent in TB patients with coughing for more than 1 month as compared with patients with a recent complaint (<1 month) (P=0.041; data not shown), probably reflecting long-term effects of immune activation on Hb concentrations. Blood coughing was present in 46 % of the cases. Although a trend could be observed, there was no significant correlation between occurrence of anaemia and blood coughing (P=0.054) (Table 2). Furthermore, anaemia was present more frequently in advanced TB (ninety-two of 118 known CXR results; 77.9 %) compared with mild or moderate TB (forty-eight of eighty-eight cases; 54.5 %) (P<0.001) as assessed by CXR abnormalities (Table 2). CXR abnormalities were classified as mild or moderate TB (n 88) or advanced TB (n 118), based on the extent of lesions on CXR as described elsewhere. Anaemia was also negatively associated with smoking habits. TB patients who were currently smoking or had ever smoked in the past (designated as ‘ever’) were surprisingly less frequently anaemic compared with those who never smoked (P=0.003) (Table 2). In a more extended study in our group we found that smoking was not associated with TB (OR 0.99 (95 % CI 0.76, 1.31); data not shown).

Anaemia also affected thirty-one control individuals, consisting of twenty-two females (10.0 %) and nine males (57.1 %). Of the female controls with low Hb (69–118 g/l), eleven individuals had a very low MCV (48.6–76.0 fl), and in the male controls with low Hb (103–128 g/l), seven individuals had a very low MCV (59.1–69.3 fl), suggesting that these individuals may have anaemia with Fe deficiency. Furthermore, nine males of the control group had a normal Hb (133–174 g/l) with a very low MCV (58.5–75.7 fl) and six females had a normal Hb (124–140 g/l) with a low MCV (60.4–74.1 fl), which could be related to thalassaemia minor. Thalassaemia (especially minor) must be considered in the differential diagnosis of normal Hb with a low MCV value.

Follow up of thirty-three tuberculosis patients: iron status indicators and anaemia

The Fe status indicators were measured in plasma for the baseline data in a random set of active TB patients before therapy (n 65) and after therapy (n 33), and compared with controls, matched for sex and age (n 76). Many indicators of Fe status were influenced by the inflammatory response. In patients with active TB, plasma Fe concentrations and Fe saturation were lower, whereas ferritin concentration was increased as compared with controls. As was to be expected, there was a strong correlation between these indicators of Fe status and CRP concentrations. Linear regression analysis showed a correlation between ferritin and CRP concentrations (R² 0.19; P<0.001) with an unadjusted coefficient of 2.9, meaning that for every 10 g/l increase in CRP concentration, ferritin concentration had increased by almost 30 m/kg/l. Moreover, at the end of TB therapy, all Fe indicators had returned to normal values with disappearance of the inflammatory response as indicated by normal concentrations of CRP and a normal ESR (Table 1). At the end of successful TB therapy, anaemia without Fe deficiency (ferritin concentrations >12 µg/l) was found in three patients (all males, Hb range 119–129 g/l, CRP all <5 g/l). One of these anaemic patients had a very low MCV and MCH value (Hb 128 g/l, MCV 60.2 fl, MCH 20.8 pg), which may suggest thalassaemia minor. On the other hand, Fe deficiency was observed in three female patients who were, however, not anaemic.

In the control group, three anaemic individuals (all female) showed a normal plasma ferritin level (35–130 µg/l). One of these controls had very low MCV and MCH values (Hb 118 g/l, MCV 63±4 fl, MCH 22±1 pg), again suggesting thalassaemia minor. A plasma ferritin value ≤12 µg/l was observed in one female control with a normal Hb value (122 g/l).

Table 2. Anaemia status of active pulmonary tuberculosis (TB) patients in relation to clinical signs or symptoms

<table>
<thead>
<tr>
<th></th>
<th>Non-anaemics*</th>
<th>Anaemics*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Coughing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>66</td>
<td>17.6</td>
<td>138</td>
</tr>
<tr>
<td>Yes</td>
<td>73</td>
<td>19.3</td>
<td>101</td>
</tr>
<tr>
<td>Chest X-ray†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild or moderate TB</td>
<td>40</td>
<td>19.4</td>
<td>48</td>
</tr>
<tr>
<td>Advanced TB</td>
<td>26</td>
<td>12.6</td>
<td>92</td>
</tr>
<tr>
<td>No wasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(18–5.8 kg/m²)</td>
<td>62</td>
<td>16.4</td>
<td>59</td>
</tr>
<tr>
<td>Mild wasting</td>
<td>37</td>
<td>9.8</td>
<td>50</td>
</tr>
<tr>
<td>(17 to &lt;18.5 kg/m²)</td>
<td>19</td>
<td>5.0</td>
<td>50</td>
</tr>
<tr>
<td>Moderate wasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(16 to &lt;17.0 kg/m²)</td>
<td>21</td>
<td>5.6</td>
<td>80</td>
</tr>
<tr>
<td>Severe wasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;16.0 kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>96</td>
<td>25.5</td>
<td>128</td>
</tr>
<tr>
<td>Never</td>
<td>43</td>
<td>11.4</td>
<td>111</td>
</tr>
</tbody>
</table>

* Criteria for anaemia: males Hb <130, females Hb <120 g/l.
† A chest X-ray was assessed in 206 patients.
‡ Subjects with BMI <18.5 kg/m² were pooled as the wasting group and compared with the no wasting group (χ² test).
**NRAMP1 gene polymorphisms and susceptibility to tuberculosis**

In the Indonesian population, the INT4 polymorphism proved to be rare and was not further analysed. The genotypes of the NRAMP1 D543N and the 3'UTR polymorphisms were in Hardy–Weinberg equilibrium in the total group of individuals as well as in the healthy controls and patients. No significant differences could be observed between healthy controls and TB patients, suggesting that NRAMP1 polymorphisms in our population are not associated with TB susceptibility. The distribution of the alleles and genotypes of the NRAMP1 polymorphisms in the Indonesian population is presented in Table 3. Furthermore, NRAMP1 polymorphisms are not associated with TB severity, as evidenced by CXR, or by anaemia in active TB (data not shown).

**Discussion**

Fe-deficiency anaemia has been reported in many developing countries\(^{19,20}\). In these countries many chronic infectious diseases are present at high rates, including pulmonary TB. The prevalence of anaemia in our healthy control group, living in a poor and TB-endemic area, was surprisingly low (6.7 %), females being more affected than males. In the present study children aged less than 15 years and pregnant women, individuals at high risk of anaemia, were not included. Like others we observed that in developing countries Fe deficiency is becoming a less important cause of anaemia compared with infection\(^{21,22}\).

It is well known that most patients with active pulmonary TB have anaemia\(^{23–25}\), but the precise mechanism remains unclear. Blood loss in the sputum (haemoptysis) has been mentioned in textbooks as one of the causes. However, original studies were never performed and haemoptysis was not associated with anaemia in the present study population. Furthermore, deficiencies of Fe and other nutrients, caused by loss of appetite and fever, are associated with a low BMI\(^{24,26,27}\).

Anaemia of chronic disease (ACD), also in active TB, is associated with a low serum Fe, Fe saturation and Fe-binding capacity, and with a high serum ferritin\(^{28–30}\), while in uncomplicated Fe deficiency serum ferritin is always low\(^{29}\). During inflammation ferritin, being an acute-phase reactant, is increased. Hence, the presence of inflammation in ACD can be estimated by increased concentrations of acute-phase protein such as CRP or by ESR\(^{30,24}\). In our population of TB patients, anaemia was mostly due to ACD and not to Fe deficiency, as shown by the comparison of haematological and Fe parameters within the same patients before and at 6 months after TB therapy, as these subjects received no Fe treatment. In ACD the decrease of Fe in the plasma compartment and the increase of ferritin, mainly in macrophages, is due to cytokine-mediated up regulation of ferritin, and reduced Fe export due to increased hepcidin production\(^{31}\). All such modifications in Fe status may be a protective response against the invading microbes\(^{3,31}\).

As the patients in the present study did not receive Fe supplementation, an increase of the Hb concentration over 6 months is mainly due to the normalisation of the inflammatory response. Alternatively, the increase of Hb concentration could be influenced by a better nutrition and a better appetite; patients gained weight as evidenced by a higher BMI after successful TB therapy\(^{26}\). Fe supplementation in developing countries, where Fe deficiency is highly prevalent, should therefore not be routinely prescribed when Fe status is unknown, as this may exacerbate infection, not only TB\(^{32}\), but also malaria\(^{33}\) or helminth infection\(^{34}\). In children living in malaria endemic areas, Fe supplementation appears to have beneficial effects in Fe-deficient children, but harmful effects in Fe-replete children\(^{35}\). In contrast, a study in Malawi reported that Fe supplementation in developing countries with a high prevalence of both HIV infection and Fe deficiency is not contraindicated\(^{36}\). Fe is believed to

**Table 3. Distribution of NRAMP1 alleles and genotypes* (Frequency and percentage frequency)**

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Allele or genotype</th>
<th>Frequency in cases</th>
<th>Frequency in controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>INT4 (469 +14G &gt; C)</td>
<td>G</td>
<td>419</td>
<td>99·3</td>
<td>704</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3</td>
<td>0·7</td>
<td>16</td>
</tr>
<tr>
<td>D543N (1627G &gt; A)</td>
<td>G</td>
<td>334</td>
<td>81·5</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>76</td>
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<td>154</td>
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<tr>
<td></td>
<td>AA</td>
<td>13</td>
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<tr>
<td>3'UTR</td>
<td>TGTG +/+</td>
<td>348</td>
<td>81·3</td>
<td>568</td>
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<tr>
<td></td>
<td>TGTG del</td>
<td>80</td>
<td>16·7</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>TGTG +/+</td>
<td>141</td>
<td>30·5</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>TGTG +/del</td>
<td>66</td>
<td>30·8</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>TGTG del/del</td>
<td>7</td>
<td>3·3</td>
<td>21</td>
</tr>
</tbody>
</table>

n.a., Not analysed.

* No significant differences were observed in the distribution of NRAMP1 single nucleotide polymorphism alleles or genotypes between tuberculosis patients and healthy controls (\(x^2\) tests).

† Genotype GA and AA combined for analysis.

‡ Genotype TGTG +/del and TGTG del/del combined for analysis.
enhance the activity of the pyrazinonic acid-containing first-line TB drug pyrazinamide\textsuperscript{37}. The conclusion that some patients with pulmonary TB and mild to moderate anaemia may benefit from Fe supplementation\textsuperscript{38,39} has no practical implication since Fe treatment of TB patients only caused a minimal increase of Hb after the first month, which disappeared after the second month of treatment. In contrast, in Fe-overload patients, Fe chelators may become important drugs for treatment of malaria, TB and HIV, to deprive micro-organisms from Fe as an essential nutrient\textsuperscript{4,38}. Fe chelators added to TB therapy should only be given to known overload patients as it may impair the host defence, and reliable investigations are not available\textsuperscript{39}.

The only effective treatment for ACD is, thus, to cure the underlying disease. Individuals in the present study with a very low MCV and only mild anaemia may have had thalassaemia minor. Haemoglobinopathies, present in 0·1–10 % of individuals of various ethnic groups in Indonesia\textsuperscript{40,41}, may be underdiagnosed\textsuperscript{42}. The risk of these individuals developing TB, however, needs to be further investigated.

Macrophages have several strategies to acquire Fe from their specific environment, including erythropagocytosis and uptake of transferrin-bound Fe, non-transferrin-bound Fe, haem and Hb. Fe is needed for both host defence and survival of the pathogen\textsuperscript{5}. There is a constant competition between host and microbes for this essential but toxic element. It remains unclear which role NRAMP1 plays in the pathogenesis of TB, being a metal transporter that resides in the phagosome membrane\textsuperscript{14}. Atkinson & Barton\textsuperscript{35} showed that NRAMP1 has a function in Fe efflux from phagosome to cytosol\textsuperscript{35}, while others found that it promotes the influx of Fe into the phagosome\textsuperscript{21}. In a recently published meta-analysis of the influence on TB susceptibility of the four most frequently studied NRAMP1 polymorphisms\textsuperscript{45} including INT4, D543N and the 3'UTR, it was shown that a large difference between populations can be observed. In Europeans none of the polymorphisms were associated with TB\textsuperscript{13}. In Africans three of the four polymorphisms (not the 3'UTR variant) were associated with TB\textsuperscript{11} and in Asians also three out of four polymorphisms (not the INT4 variant) were associated with TB\textsuperscript{46,47}. A striking difference between the present study and the other studies in Asian populations is that the allele frequencies of the polymorphisms are very different. We find the C allele of the INT4 polymorphism in only 2 % of our controls while this is found in 14 % of Japanese and 21 % of Chinese controls. We find the 3'UTR deletion allele in 22 % of our controls while this is found in 8–19 % in five other Asian populations. Similarly we find the A allele of the D543N polymorphism in 22 % of our controls while this is found in 2–15 % of six other Asian populations. It appears that not only the association of TB with NRAMP1 polymorphisms is different between continents but also the distribution of alleles is also very different between Asian populations, which may reflect a difference in selective pressure in the past. If the NRAMP1 polymorphisms studied here are functional polymorphisms influencing TB susceptibility directly, we should have observed a similar association. If these polymorphisms are, however, merely in linkage disequilibrium with a functional variant elsewhere in NRAMP1 (or a neighbouring gene) the association between certain alleles and TB susceptibility can vary greatly between populations as has been shown by Li et al.\textsuperscript{45}. Further studies into other variations in NRAMP1 or in other genes involved in susceptibility to TB are needed.

To conclude, the present results support earlier observations that anaemia in active TB is mainly the result of ACD, more than of Fe deficiency. As the Hb concentration increased after successful TB therapy, Fe supplementation was not necessary. Fe supplementation in developing countries should be restricted to children and women of reproductive age, who have the highest prevalence of Fe deficiency.

### Acknowledgements

The present study is part of the project ‘Immunogenetic basis of susceptibility to and disease manifestations of mycobacterial infections’, financially supported by the Royal Academy of Arts and Sciences (KNAW), The Netherlands. We thank Maya Anugrah and Erita Istriana for their assistance in the clinical work in PPTI Jakarta under supervision of Dr Halim Danusantoso. We thank Dr Dorine Swinkels for her support and fruitful discussion.

### References


