ORIGINAL RESEARCH ARTICLE

Assay of Serum Iron and TIBC: A Preliminary Study for Survey Shortlisting Suspected Tuberculosis Patients

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Abstract

Aim: *Mycobacterium tuberculosis* (*Mtb*) growing within the phagosome of macrophases secretes siderophores, a small molecule having a high affinity for Fe^{+3} iron, to take up iron-loaded mycobactin (MBT) and carboxymycobactin (CMBT) from the environment to meet its iron (Fe) need. *Mycobacterium tuberculosis* is well capable to utilize Fe from heme and hemoglobin by the secretion of heme-binding protein, cell surface proteins, etc., by the *mycobacteria*. On the other hand, the measurement of serum total iron binding capacity (TIBC) denoting the maximum amount of Fe carried by transferrin (Tf) present in serum entails indirectly a measure of serum Tf level. The index author has interpreted the serum iron and serum TIBC level and the ratio of serum iron and serum TIBC as a preliminary survey to shortlist the suspected population deserving confirmatory test for tuberculosis (TB). This is to categorically declare that assay of these parameters is not to be used as TB diagnostic but only for shortlisting suspected TB patients from the general population.

Materials and methods: The study was conducted on total of 180 participants divided into 3 groups: Group I - normal control ($n = 45$); Group II - lung disease control ($n = 45$); and Group III - patients suffering from TB (3A: Pulmonary TB ($n = 45$) and 3B: Extrapulmonary TB ($n = 45$)). Serum Fe and TIBC levels were measured for all participants and also for group III and group II subjects after one month with the usual treatment. The level of significance was assessed using Student's *t*-test. All the subjects in this study had normal liver function tests and they did not suffer from iron overload diseases or any malabsorption of iron syndrome.

Result: At baseline, serum Fe was significantly high in TB patients whereas serum TIBC was significantly decreased. After one month's additional anti-TB (A-TB) drug treatment serum iron had increased but not significantly ($p = 0.15$) and serum TIBC had increased significantly ($p = 0.04$). Statistical computation of the ratio of serum Fe and serum TIBC in TB patients had shown to be as high as 0.63, and more than that.

Conclusion: From statistical computation, it might be conferred that serum Fe more than 149 µg/dL and the ratio of serum Fe to serum TIBC more than 0.63 (which is more important) in preliminary survey detecting TB patients would shortlist the TB suspects deserving confirmatory test for TB diagnosis.

Keywords: Carboxymycobactin, Mycobactin, Siderophores, Superoxide dismutase, Transferrin.

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Introduction

Globally, in 2019 an estimated 10 million people fell ill with tuberculosis (TB) and there was an estimated total of 1,418,000 TB-related death.[1] About 7.1 million persons globally were reported to have been newly diagnosed and notified in 2019 with still a large gap (2.9 million) between the number of newly diagnosed and reported and the estimated population to have developed TB in 2019.[1] This gap is due to a combination of underreporting and non-diagnosis of TB subjects. Maclean E et al., had inferred TB biomarker discovery studies to be poorly designed and findings not confirmed by independent studies; and sought more validation studies to consider intended diagnostic use.[2] Chattopadhyay DK, had indoctrinated detection of L-methionine-S, R-sulfoximine (MSO) sensitive serum glutamine synthetase (GS) as a diagnostic marker for pulmonary TB (PTB) and extrapulmonary TB (EPTB).[3] The same author had demonstrated highly elevated sodium cyanide (NaCN)-resistant serum superoxide dismutase (SOD) as a diagnostic marker for TB.[4] Chattopadhyay DK had also endorsed inhibited serum cholinesterase (ChE) activity as a reliable diagnostic aid for TB.[5]

These diagnostics are not too expensive but require the utility of sophisticated apparatus and trained personnel. So, the need of the hour in developing countries like India is to adopt simpler tests for preliminary surveys to decrease sample size, and to go for diagnostics of TB for the shortlisted population. For *mycobacteria*, iron is the cofactor of enzymes like SOD, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; enzymes involved with tricarboxylic acid cycle, pyrimidine synthesis, etc. numbering for at least 40 enzymes as encoded in its genome.[6] In mammals, most serum iron circulates bound to transferrin, glycoprotein, or to lactoferrin.[7] Reduction of pH of the vesicle to 5.5 by hydrogen ion proton pump (H^+ATPase) causes dissociation of iron-bound transferrin vesicles to release its iron ion.[8] Macrophages are a suitable favorable sites to acquire iron as macrophages by their specific cell surface receptors for transferrin, lactoferrin, hemoglobin-haptoglobin can internalize

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iron from senescent erythrocytes by high iron flux due to recycling.[9] To meet the iron needs, *Mtb* excretes out siderophores; mycobactin (MBT), and carboxymycobactin (CMBT); small molecules with high affinity for Fe^{+3}.[10] These siderophores can scavenge iron from transferrin and lactoferrin, the host proteins.[11] Heme and hemoglobin are well utilized by *Mtb* as iron sources.[12]

Several proteins such as heme-binding protein, cell surface protein, and RND efflux pumps had been reported in heme utilization by *Mtb*.[13–15] Again, transferrin iron-binding capacity or total iron-binding capacity (TIBC) is a laboratory test measuring the capacity of blood to bind iron with transferrin. As transferrin remains the most dynamic iron carrier, serum TIBC indirectly measures transferrin level in blood as the maximum amount of iron it can carry.[16] With the presence of abundant Fe^{+3}-chelator siderophores which are stronger binding sites for iron as well as diffusible ones, a major decompartmentalized state of iron ensues in host tissues. The process of this decompartmentalization of iron in host tissues might get exaggerated with the discernible lowered level of serum transferrin in TB patients due to infection as well as protein-energy malnutrition. In that scenario, it was contemplated that serum iron concentration in TB patients might be higher than serum TIBC which is the maximum serum iron concentration that could be carried by transferrin with its full saturation. With that eventuality, it was logical to assay serum iron and serum TIBC from the general population for a preliminary survey for shortlisting TB suspects. It was also logical to assay the same parameters after one month with anti-TB drugs to identify the effect of drug treatment on serum iron and serum TIBC. It had also been mulled in this study to interpret serum iron and serum TIBC along with the ratio of these parameters at baseline to authenticate any possible preliminary survey for shortlisting suspected TB patients from the general population as such.

In this study, consolidated standards of reporting trials (CONSORT) encompassing various initiatives was adopted. Notwithstanding, it is an evidence-based study having a minimum set of recommendations for reporting randomized control trials (RCT). It is intended to report in a full and clear manner facilitating quality assessment.

Materials and Methods

The cohort study was conducted at B S Medical College and Hospital, Bankura – 722 102, West Bengal, India. The study protocol to collect blood samples from human subjects was approved in writing by Institutional Ethics Committee (vide memo no 3/BSMC/04 dt. 14-05-2004). The purpose of the study was explained to all participants and before the collection of blood-informed verbal consent was obtained from each of the subjects. For under-aged participants verbal consent was sought and obtained from their legal guardians before collection of blood. A total of 180 participants aged 4–66 years were enrolled. The study was conducted in three phases that are in May–June, 2007; Feb–March, 2008; and Jan–Feb, 2009. In each phase, 60 participants were enrolled. The subjects were divided into three groups (Gp):

Group-I: Normal control subjects ($n = 45$) – healthy relatives of TB patients having no clinical signs, or symptoms, suggestive of TB, or any sort of disease were considered for this group. They were sputum negative for acid-fast bacilli (AFB).

Group-II: Lung disease control subjects ($n = 45$) – included patients suffering from respiratory tract infection or bronchiectasis or bronchial asthma or bronchogenic carcinoma. The subjects were selected from patients attending outpatient department (OPD) of B S Medical College and Hospital, Bankura.

Group-III: Tuberculosis subjects–Patients suffering from TB attending OPD of above-mentioned hospital and also being admitted to the Isolation Ward of the same hospital were taken into account. For these TB patients anti-TB drug therapy was started between 0 and 15 days. Based on prior diagnosis, TB subjects were categorized as **Subgroup IIIA**: Patients suffering from PTB ($n = 45$); and **Subgroup IIIB**: Patients suffering from EPTB ($n = 45$). Extrapulmonary tuberculosis patients included TB - lymphadenitis, pleural effusion, meningitis; lupus vulgaris; and also spinal, hip joint, intestinal, urinary bladder, and miliary TB. Thus the total number of participants was $45 \times 4 = 180$. They were assayed in three phases as already described, so in each phase, $180 \div 3 = 60$ participants were enrolled. Tuberculosis patients were diagnosed clinically by characteristic symptoms and signs as well as by other investigative procedures like radiological study, sputum for AFB, fine needle aspiration and cytology whenever possible, ELISA technique for serodiagnosis; and detection of lipoarabinomannan (secreted antigen of *mycobacteria*) in serum and urine.[17] Multidrug resistant TB patients were not included in this study. It was the exclusion criterion for the study regarding TB patients concerned. It is noteworthy to mention that all the subjects had normal liver function tests. It was an important inclusion criterion for this study as transferrin, the iron-binding protein, is synthesized predominantly in the liver. In this survey, subjects suffering from hereditary hemochromatosis, i.e. iron overload disease were not included. Also excluded were the patients suffering from impaired iron transporter diseases. That was an important exclusion criterion for this research study.

Collection of Blood: Morning blood samples were collected from the subjects by venipuncture. Blood samples were left to clot; serums so obtained were transferred in clean and sterile Ependorf tubes and kept in a refrigerator at 2–4°C until assayed on the same day of sample collection. After the assay, the remaining serum was stored at –20°C if it might necessitate repeating the assay procedure. Repeat blood collection was done for Group-II and Group-III subjects after 30 days with the usual treatment. During these additional 30 days, TB patients were kept under the directly observed treatment (DOT) program under the Revised National Tuberculosis Control Program.

Estimation of Serum Iron (normal reference value 60–170 µg/dL): Serum protein was precipitated with a reagent containing hydrochloric acid (to dissociate iron), thioglycolic acid (to reduce iron) and trichloroacetic acidto precipitate protein). Iron in a ferrous state reacts with chromogen (sodium acetate in ferrozine) to develop pink colored complex, which is measured colorimetrically.[18]

Estimation of TIBC (normal reference value 240–450 µg/dL): To measure TIBC, serum was treated with iron standard (ferric chloride), and the excess iron was removed by adsorption with excess magnesium carbonate ($MgCO_3$). The iron concentration of iron-saturated serum is the measurement for TIBC.[19]

Statistical Analysis

Statistical analysis of the results was made by using Statistical Software for Social Sciences (SPSS version 21.0). The level of significance was assessed using an independent Student's *t*-test. $p < 0.05$ was considered to be significant statistically.

Table 1: Baseline serum iron and TIBC and also after 30 days' additional treatment

	Serum iron (µgm/dL)			*Serum TIBC (µgm/dL)*		
Subjects	*At baseline*	*After 30 days*	*p-value*	*At baseline*	*After 30 days*	*p-value*
Normal control ($n = 45$)	100.2 ± 10.1	—		325.1 ± 14.8	—	
Lung disease control ($n = 45$)	112.7 ± 6.5	119.8 ± 4.7	$p = 0.5$	312.7 ± 12.8	321.0 ± 9.8	$p = 0.4$
Tuberculosis patients						
A) Pulmonary ($n = 45$)	192.8 ± 14.6	238.3 ± 21.8	$p = 0.15$	187.6 ± 15.4	231.1 ± 13.1	$p = 0.04^*$
B) Extrapulmonary ($n = 45$)	190.2 ± 13.7	231.4 ± 16.9	$p = 0.15$	180.7 ± 18.8	221.2 ± 15.3	$p = 0.04^*$

NB-,*denotes that *p* is significant

Result

Baseline serum Fe in PTB and EPTB patients showed a significant increase when compared with those of normal control ($p = 0.004$) and lung disease control ($p = 0.005$) subjects. While there was no noteworthy change in the level of serum iron after 30 days' usual treatment in lung disease control subjects ($p = 0.5$); there was a noteworthy increase of serum Fe in TB subjects after 30 days' A-TB drug therapy ($p = 0.15$). On the other hand, initial or baseline serum TIBC in TB patients had reported a significantly decreased value ($p = 0.003$ against normal control and $p = 0.004$ against lung disease control subjects).

While after 30 days' usual treatment, lung disease control subjects showed little increase in serum TIBC ($p = 0.4$); TB patients had a significant increase in serum TIBC ($p = 0.04$) after 30 days' A-TB drug therapy. It is to mention that in the estimation of serum TIBC, after saturating serum with iron standard, excess iron is removed by adsorption on $MgCO_3$.

In this process, the siderophore-Fe complex present in the serum of TB patients is also adsorbed on $MgCO_3$ as the complex has lower molecular weight and thus is precipitated out from the test solution.

With the result as depicted in Table 1, the statistical computation was looked up to engross the lowest cut-off value for serum Fe and the highest cut-off value for serum TIBC which can be used as a preliminary survey shortlisting suspected TB patients.

Now statistically, the area under the normal curve between ($\overline{X}$–3SD) and ($\overline{X}$+3SD) is 99.74% of the total area under normal curve where $\overline{X}$ is mean value and SD is the standard deviation. The conventional method to determine a cut-off is the 99.7% confidence interval (CI) of the mean, a crude measure for observing cut-off values. An interval; mean ±3 SD obtained by subtracting 3 × SD from the mean and by adding 3 × SD predicts that the chance of a test value coming outside this interval will be less than 0.3%. For parameters showing higher values, mean – 3 SD had been taken as cut off value, and for parameters having lower values, mean +3SD had been presumed as cut off value. As serum Fe is markedly elevated in TB patients, values depicted in the left half of the normal curve have been considered for statistical computation and 3 times of SD value has been subtracted from the mean value. On the contrary, serum TIBC in TB had shown marked inhibition and for statistical computation, right half of the normal curve has been considered and three times of SD value has been added to the mean value. Similarly, for computation for normal control and lung disease control subjects, the right half of the normal curve has been considered for serum Fe and the left half of the same curve for serum TIBC. Now, if from a mean of baseline serum Fe level of TB patients is deducted three times of SD, serum iron in PTB subjects is 149.0 µg/dL, and in EPTB subjects 149.1 µg/dL. In normal and lung disease control subjects with three times SD added to the mean, figures are 130.5 µg/dL and 132.2 µg/dL respectively for serum Fe. Also, if three times SD is added with mean, serum TIBC is calculated as 233.8 µg/dL and 237.1 µg/dL respectively in PTB and EPTB patients. With a deduction 3 times of SD from the mean, serum TIBC becomes 280.7 µg/dL and 274.3 µg/dL respectively in normal and lung disease control subjects. To circumvent the amount of serum Fe against the capacity of transferrin to carry it (serum TIBC), the ratio of serum Fe and serum TIBC has been calculated for each group of subjects. This ratio is 0.46 in normal control, 0.48 in lung disease control, 0.64 in PTB, and 0.63 in EPTB subjects. Noteworthy to mention, while computing for cut-off values of contrasting levels of parameters in TB patients and normal control as well as disease control subjects, there were neither any coincidences nor any cross-over of cut-off values.

Discussion

Let the author begin a discussion in relation to statistical analysis of the autopsy report in the year 1920 from South Africa. It revealed a great relationship between high macrophage iron stores and death from TB.[20] Macrophages have the characteristics of having high iron flux enabled by recycling of Fe from senescent RBCs and also by specific cell surface receptor-mediated iron internalization of transferrin, lactoferrin, and haemoglobin-haptoglobin.[9] It is logical that siderophore by its efficient capacity to capture Fe from host-specific iron-binding proteins promotes the growth of intracellular mycobacteria by providing a source of iron.[21] It was reported that *Mtb* does encode two iron storage proteins; BfrA, a bacterioferrin, and BfrB, a ferritin-like protein.[22] By using IrtA/IrtB transporters *Mtb* might take up iron-bound siderophores.[23] This IrtAB mediates the reduction of Fe^{+3} in the internalized ferric-siderophore complex into Fe^{+2} and its release.[24] The iron-free siderophores (desferricarboxymycobactin) are exported and recycled through the inner membrane with the help of mycobacterial membrane proteins (MMPs) bound to transporter complexes.[25] Recycling of desferricarboxymycobactin enables *Mtb* to acquire iron at a low metabolic cost.[26] Genetic disruption of the recycling process reduces the capacity of *Mtb* to take up iron and causes siderophore-mediated self-poisoning.[26] It demonstrates that mycobacteria virulence is dependent on its essential role of iron acquisition by siderophore.[27] *Mycobacterium tuberculosis* cannot use iron salts in the absence of siderophores and inactivation of siderophore export and recycling reduces the capacity of *Mtb* to take up iron.[26] There was decreased *Mtb* growth in macrophages with a mutant defect in mycobactin synthesis.[27] Also, the irtAB mutant being unable to utilize iron from Fe-carboxymycobactin had reported defective *Mtb* growth.[23] It might sound logical that an assay of serum siderophore might have a value as a diagnostic predictor of mycobacterial disease.

But, the presence of CMBT in infected tissue or body fluid is yet to be demonstrated and is not suited for diagnostic use.[28] Varghese GM et al. had sought more studies to authenticate siderocalin (siderophore binding protein secreted by host cells in response to TB infection to limit access of *Mtb* to iron) as a prospective potential marker of active TB as detailed.[29] So, an assay of siderophore in serum is neither feasible nor established potential utility as a diagnostic marker of active mycobacterial disease.

Mycobacterium tuberculosis within phagosome interacts with early endosomes and does not acidify below pH 6.3–6.5.[30]

Thus, by preventing phagosome acidification and lysosomal fusion, *Mtb* is expected to evade host defense and acquire Fe from host endosomal holo transferrin. The TB patients in this study have recorded significantly higher serum iron levels. Also to mention, the subjects of this study hail from an area where the iron level in underground drinking water often exceeds the maximum permissible limit. So, iron loading might increase mycobacterium replication as evidenced by in vitro and animal model studies.[31] Transferrin, the glycoprotein of molecular weight of about 80KDa, has two specific high-affinity Fe^{+3} binding sites. Major subgroups of transferrin in humans are (a) serum transferrin (siderophilin, serotransferrin or β metal-binding globulin) being produced in hepatocytes and to some extent in choroid plexus, is found in serum, cerebrospinal fluid, and semen. (b) lactoferrin (lactotransferrin or milk red protein) being synthesized in mucosal epithelial cells, was found first in milk but has since been found in tears, saliva, and neutrophil leucocytes.

Iron after being absorbed as Fe^{+2} form reacts with apotransferrin forming an intermediate which on being oxidized by molecular oxygen gives stable Fe^{+3}-transferrin-carbonate complex.[32]

In this reaction, three protons (H^+) are released per Fe^{+3} ion bound. It is interesting to note that this released H^+ ion can be well-utilized by *Mtb* for a dismutation reaction catalyzed by iron-cofactored SOD secreted by it and thus providing soluble oxygen for the survival of the aerobe. Cronje L et al. had shown that experimental supplementation of iron to macrophage culture or in experimental animals infected with *Mtb* had enhanced multiplication of the organism.[31] For the survival of *Mtb* in macrophages, siderophore-mediated iron uptake is essential as knockout mutants could not survive inside macrophages in low iron conditions as the said mutants were defective in siderophore synthesis and uptake. From the statistical computation of the results in this study, the ratio of serum iron and serum TIBC for normal control and lung disease control subjects amounts to 0.46 and 0.48 respectively at the maximum. That denotes that serum transferrin is saturated with iron to the maximum extent of 46 and 48% in normal control and lung disease control subjects respectively. Transferrin, in fact, is a part of the innate immune system by preventing the formation of reactive oxygen species and also by chelating free toxic iron, it impedes bacterial survival. On the other hand, by statistical computation, it is found that the concerned ratio in TB patients is 0.63 at the minimal. This high degree of iron saturation of transferrin in TB patients might sound fallacious. But, the fact is that siderophore plays a major role in chelating serum iron and also becomes major transporter of serum iron in TB patients as already elaborated. So, a significant increase in serum iron level is recorded in TB patients. Ironically, while measuring serum TIBC in TB patients, only the maximum amount of iron carried by serum transferrin itself is reflected in the result as the siderophore-iron complex gets adsorbed on $MgCO_3$ due to its lower molecular weight, as had been explained in the result section. Thus, serum TIBC in TB patients reflects only the maximum amount of iron carried by transferrin itself and understandably records a significantly decreased value. So, a significantly higher value for the concerned ratio is recorded in TB patients. Thus, siderophores play a vital role in the pathogenesis of TB as chelators of Fe^{+3} ion bound to host transferrin and releasing iron into host cells after the reduction from Fe^{+3} to Fe^{+2}. With infection caused by *Mtb,* there is decompartmentalization of iron in host tissues by binding of Fe^{+3} to siderophores which are stronger binding sites as well as diffusible ones. In the decompartmentalized state of iron, iron-catalyzed oxidation of thiol groups results in the formation of highly reactive superoxide ($O_2^{\cdot}$) Ironically, this $O_2^{\cdot}$ is again used up by *Mtb* by dismutation reaction to generate soluble oxygen for its survival. Measurement of TIBC indirectly indicates serum transferrin level. There is also a mathematical equation to calculate serum transferrin from TIBC. This is advantageous as the measurement of TIBC is less expensive than direct measurement of transferrin. Serum TIBC in TB patients has shown significantly lower values. Lower TIBC, i.e. lower serum transferrin in TB patients is due to infection and protein malnutrition. Transferrin by chelating free toxic iron and thus acting as a protective scavenger exacerbates a pivotal role to play in the innate immune system. With an increasing degree of inflammation, serum transferrin level falls and the stage of major decompartmentalization of iron ensues. The increase in serum TIBC in TB patients after 30 days' A-TB drug therapy is due to a decrease in the degree of infection (decrease in mycobacterial load) and hence in extracellular siderophore level. On the other hand, an assertive and veritable increase in baseline serum iron in TB patients was due to the abundant export of extracellular high-affinity iron binding siderophores by *Mtb.* Higher efficiency of siderophore-mediated uptake of iron ions from heme and non-haem protein and also from hemoglobin arising out of extensive tissue damage and airway bleeding in TB patients specially PTB patients; indicates that this is the default pathway primarily used by *Mtb*.[26] It is interesting that siderophores are capable to scavenge iron from mineral phases (iron oxide and hydroxides) by the formation of soluble Fe^{+3} complexes that can be taken up by active transport mechanisms.[33] After 30 days of additional A-TB drug therapy, serum iron in TB patients had increased but not significantly ($p = 0.15$). This increase in serum Fe can be acclaimed as with A-TB drug therapy, the decompartmentalization state of iron in the body begins to revert back to the usual compartmentalized state of iron and hence higher serum Fe level. It is logical that most of the serum Fe^{+3} ion is bound to *Mtb*-secreted siderophores instead of serum transferrin as it is the stronger scavenger of Fe^{+3} ions. It has become reflected in the result section of the study showing the decreased levels of serum TIBC in TB patients. The increase of serum Fe in TB patients is due to the presence of increased siderophore-bound Fe^{+3} complex. Serum TIBC measures only iron bound to transferrin, which records an inhibited result in TB patients. Now, from the plausible computation of the parameters, as done in the Result section, it may be interpreted that in preliminary study for survey detecting TB patients; if serum Fe is more than 149 µg/dL and the ratio between serum Fe and serum TIBC is more than 0.63 (which is more important) for an individual, he or she becomes positive for this preliminary study.

For evaluating the outcome of this preliminary study to shortlist the suspected TB patients as well as subjects having latent TB; the receiver-operating characteristic (ROC) analysis was adopted. So,

using SPSS software, the corresponding empirical ROC curve was drawn by the non-parametric method. The curve as well as the corresponding area (AUC) had established that the cut-off values for serum iron and serum TIBC and also the concerned ratio of those two had predictive values to shortlist the suspected TB patients from the general population as such.

To assay serum Fe and TIBC as a sample study might be a preliminary survey to shortlist individuals deserving diagnostics for TB. Demonstration of *Mtb* origin MSO sensitive serum GS; *Mtb* origin NaCN resistant enhanced serum SOD and inhibited host origin serum ChE will clinch diagnosis for TB.[5] With this preliminary field survey and then confirmatory tests to diagnose TB as detailed above might be an effective weapon to keep number of non-diagnosed TB patients to almost baseline. This preliminary survey can be accomplished by field health workers collecting blood samples from individuals at their residences itself.

The author categorically confesses some of the limitations of this work. The liver is the main site for the synthesis of transferrin. So, a normal liver function test was an important criterion to select test subjects. In this survey, subjects suffering from iron overload diseases (hereditary hemochromatosis, heterozygotes, and homozygotes) should be excluded. Patients suffering from malabsorption of iron like celiac disease, tropical sprue, Crohn's disease, etc., and also with impaired nutrient transporter (postmucosal condition) diseases like intestinal lymphangiectasia, macroglobulinemia, etc. should be excluded.

Conclusion

With all sincerity, it is to declare that this research work is not meant for as a TB diagnostic. It is limited only to a preliminary survey for shortlisting suspected TB patients. The patients having a higher ratio of serum iron and serum TIBC at baseline as explained in the Discussion must be subjected to confirmatory TB diagnostics as clarified in this article. It's a great solemnity to promulgate that this preliminary survey is exquisite and unequivocal to shortlist individuals who deserved to undergo confirmatory tests for TB diagnosis. This will facilitate early diagnosis of TB patients rendering instantaneous endorsement of A-TB drug therapy. This might fulfill the accelerator campaign to National Strategic Plan in India - *TB* Harega Desh Jeetega (TB will be defeated, the Nation will triumph).

Clinical Significance

As a preliminary survey for shortlisting suspected TB patients; this research work has an immense potential as a field study in developing and under-developed countries. The collection of samples is easy and also the methodology is not complicated as well as not expensive. Though the TB diagnostics had already been elaborated on in this Paper, it is not irrelevant to mention the recent publication of this author where the measurement of the ratio of serum SOD and whole blood glutathione peroxidase had also been put forward as TB diagnostic.[34] It is also noteworthy to refer to the very recent publication of the index author, where it had been inferred that oral zinc (Zn) supplementation (25 mg of elemental Zn daily) in TB patients had augmented the process of the decompartmentalized state of iron reverting back to a normal compartmentalized state of Fe and thus by curtailing superoxide formation and hence dismutation reaction and thus preventing the formation of soluble oxygen required for survival of *Mtb* having Fe-cofactored SOD; and therefore ultimately had enhanced the killing of *Mtb*.[35]

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