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Analytical evaluation of serum non–transferrin-bound iron and its relationships with oxidative stress and cardiac load in the general population

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Abstract

Excessive iron accumulation provokes toxic effects, especially in the cardiovascular system. Under iron overload, labile free non–transferrin-bound iron (NTBI) can induce cardiovascular damage with increased oxidative stress. However, the significance of NTBI in individuals without iron overload and overt cardiovascular disease has not been investigated. We aimed to examine the distribution of serum NTBI and its relationship with oxidative stress and cardiac load under physiological conditions in the general population.

We enrolled individuals undergoing an annual health check-up and measured serum NTBI and derivatives of reactive oxygen metabolites (d-ROM), an oxidative stress marker. In addition, we evaluated serum levels of B-type natriuretic peptide (BNP) to examine cardiac load. We excluded patients with anemia, renal dysfunction, cancer, active inflammatory disease, or a history of cardiovascular disease.

A total of 1244 individuals (57.8 ± 11.8 years) were enrolled, all of whom had detectable serum NTBI. d-ROM and BNP showed significant trends across NTBI quartiles. Multivariable regression analysis revealed that serum iron and low-density lipoprotein cholesterol were positively associated with NTBI but that age, d-ROM, and BNP showed an inverse association with this measure. In logistic regression analysis, NTBI was independently associated with a combination of higher levels of both d-ROM and BNP than the upper quartiles after adjustment for possible confounding factors.

Serum NTBI concentration is detectable in the general population and shows significant inverse associations with oxidative stress and cardiac load. These findings indicate that serum NTBI in physiological conditions does not necessarily reflect increased oxidative stress, in contrast to the implications of higher levels in states of iron overload or pathological conditions.

Abbreviations: BNP = B-type natriuretic peptide, BP = blood pressure, d-ROM = derivatives of reactive oxygen metabolites, FPG = fasting plasma glucose, LDL-C = low-density lipoprotein cholesterol, NTBI = non–transferrin-bound iron, ROS = reactive oxygen species, UIBC = unsaturated iron-binding capacity.

Keywords: cardiac load, general population, non–transferrin-bound iron, oxidative stress, physiological condition

1. Introduction

Iron is an abundant metal and an essential micronutrient for maintaining the body homeostasis.[1,2] Most iron exists intracellularly, bound to hemoglobin in red blood cells or stored as cytoplasmic protein ferritin in hepatocytes and macrophages within the liver and spleen.[1,2] Serum iron (Fe) is bound mainly to the carrier protein transferrin, and its circulating concentration in physiological states is controlled within constrained limits. Thus, circulating transferrin is not entirely saturated with Fe, and more than 50% of transferrin is unbound to iron, at least under healthy conditions.[1,2]

When the iron-overload state is provoked in patients with hematopoietic diseases such as thalassemia and myelodysplasia, labile free non–transferrin-bound iron (NTBI) will be detectable with transferrin saturation >70% to 80%.[1–3] Under such pathological conditions, increased NTBI augments production of reactive oxygen species (ROS) through the Fenton and Haber–Weiss reactions. In these cases, iron toxicity complicated by excess free radical generation exerts harmful effects on various tissues and organs.[1–3] In patients with both heart failure and iron-deficiency anemia, iron supplementation improves heart failure symptoms.[4–6] In contrast, redundant administration of iron may increase oxidative stress through the production of ROS and lead to excessive iron accumulation in the myocardium and

Editor: Masood Sepehrimanesh.

The authors have no conflicts of interest to disclose.

Supplemental Digital Content is available for this article.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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How to cite this article: Sugiura T, Dohi Y, Takase H, Fujii S, Seo Y, Ohte N. Analytical evaluation of serum non–transferrin-bound iron and its relationships with oxidative stress and cardiac load in the general population. Medicine 2021;100:7(e24722).

Received: 12 September 2020 / Received in final form: 18 December 2020 / Accepted: 19 January 2021

http://dx.doi.org/10.1097/MD.0000000000024722

hemochromatosis.[4–6] Moreover, sustained circulating iron overload could promote atherosclerosis with increased NTBI via induction of endothelial damage.[7–9]

Extracellular NTBI can induce cardiovascular damage in iron-overload states, whereas the significance of NTBI in individuals without iron overload and overt cardiovascular disease has not been investigated. In the present study, we aimed to examine the relationships of NTBI with oxidative stress and cardiac load in the general population.

2. Methods

For this study, individuals attending a periodic physical check-up were enrolled. The study protocol was approved by the ethics committees of Enshu Hospital, and the study was performed in accordance with the principles of the Declaration of Helsinki. Each participant gave written informed consent before the examination.

2.1. Participants

Individuals who visited Enshu Hospital in 2015 for a health check-up were screened ($n=1400$) for their eligibility to participate in the present study. We excluded patients with anemia (hemoglobin <10 g/dL), taking iron medications, renal dysfunction (estimated glomerular filtration rate <30 mL/min/1.73 m^2), cancer, active inflammatory disease, or a history of cardiovascular events (stroke, myocardial infarction, and heart failure), or with obvious ST segment or T wave abnormality, Wolff–Parkinson–White syndrome, pacemaker implantation, or frequent arrhythmia (including atrial fibrillation and atrial flutter) in the standard 12-lead electrocardiogram. The data for the remaining 1244 patients were used for the current analysis.

For laboratory measurements, we took blood samples early in the morning after an overnight fast and measured serum NTBI. For systolic and diastolic blood pressure (BP) measurements, we used the non-dominant arm and a validated oscillometric technique (HEM-7070; Omron Corporation, Kyoto, Japan) with the patient in a seated position. Of three consecutive BP measurements taken at 2-min intervals, we recorded the mean of the second and third measurements as the BP value, because BP fell markedly with repeating measurements and reached almost plateau at second to third measurement. Patients who were taking antihypertensive medications or with systolic BP ≥140 mmHg and diastolic BP ≥90 mmHg were defined as having hypertension.[10] Those taking lipid-lowering medications or with high-density lipoprotein cholesterol <40 mg/dL, low-density lipoprotein cholesterol (LDL-C) ≥140 mg/dL, or triglycerides ≥150 mg/dL were defined as having dyslipidemia.[11] Patients taking blood glucose–lowering medication or presenting with fasting plasma glucose ≥126 mg/dL were identified as having diabetes.[12] The estimated glomerular filtration rate was calculated using a modified formula from the Modification of Diet in Renal Disease study for the Japanese population.[13]

2.2. Biochemical analysis

We used standard laboratory assays for all biochemical tests, including determination of serum total cholesterol, LDL-C, high-density lipoprotein cholesterol, and triglycerides. Measurement of serum NTBI concentration was performed with automated systems, as previously described.[14] Levels of Fe and unsaturated iron-binding capacity (UIBC) were quantified using commercial kits (QuickAuto Neo Fe and QuickAuto Neo UIBC, Shino-Test). Transferrin saturation was calculated by 100 × Fe/(total iron-binding capacity), in which total iron-binding capacity indicates the sum of Fe and UIBC, and was expressed as a percent value of the total. Serum levels of derivatives of reactive oxygen metabolites (d-ROM) were measured to evaluate oxidative stress, as described previously.[15] Briefly, serum samples were mixed with a buffered solution, and a chromogenic substrate was added to the mixture. Samples were immediately incubated in the analyzer for 5 minutes, after which absorbance was recorded at 505 nm, with d-ROM levels expressed in Carratelli units. Plasma B-type natriuretic peptide (BNP) levels were determined using a commercially available chemiluminescence enzyme immunoassay (MI02 Shionogi BNP kit; Shionogi, Osaka, Japan).

Table 1

Characteristics of the study participants ($n=1244$).

Variable	All participants ($n=1244$)
Age (years)	57.8 ± 11.8
Male sex, n (%)	801 (64.4)
Body mass index (kg/m^2)	22.6 ± 3.1
Current smoker, n (%)	243 (19.5)
Systolic BP (mmHg)	123 ± 14
Diastolic BP (mmHg)	74 ± 9
Pulse rate (bpm)	63 ± 9
Hemoglobin (g/dL)	14.0 ± 1.2
AST (U/L)	20.5 ± 4.9
ALT (U/L)	18.8 ± 7.2
γ-GTP (U/L)	28.5 ± 14.1
Creatinine (mg/dL)	0.79 ± 0.16
HDL-C (mg/dL)	60.9 ± 16.0
LDL-C (mg/dL)	123.2 ± 27.6
Triglycerides (mg/dL)	103.4 ± 53.6
FPG (mg/dL)	96.5 ± 16.3
Fe (μg/dL)	107.5 ± 36.4
UIBC (μg/dL)	216.1 ± 56.7
TSAT (%)	33.8 ± 12.0
Ferritin (ng/mL)	113.6 ± 82.8
NTBI (μmol/L)	0.193 ± 0.077
d-ROM (Carratelli units)	355.4 ± 58.8
BNP (pg/mL)	15.7 [8.4–27.9]
Cardiovascular risk factors	
Hypertension, n (%)	383 (30.6)
Dyslipidemia, n (%)	613 (49.3)
Diabetes mellitus, n (%)	92 (7.4)
Obesity, n (%)	237 (19.1)
Medications	
ACE inhibitor or ARB, n (%)	173 (13.9)
β-blocker, n (%)	18 (1.4)
Calcium channel blocker, n (%)	193 (15.5)
Diuretics, n (%)	22 (1.8)
Lipid-lowering drug, n (%)	177 (14.2)
Hypoglycemic drug, n (%)	69 (5.5)
Antithrombotic agent, n (%)	29 (2.3)

Data are presented as the mean ± standard deviation, as *n* (%), or median [interquartile range]. ACEi = angiotensin-converting enzyme inhibitor, ALT = alanine transaminase, ARB = angiotensin receptor blocker, AST = aspartate transaminase, BNP = B-type natriuretic peptide, BP = blood pressure, CCB = calcium channel blocker, d-ROM = derivatives of reactive oxygen metabolites, FPG = fasting plasma glucose, HDL-C = high-density lipoprotein cholesterol, LDL-C = low-density lipoprotein cholesterol, NTBI = non–transferrin-bound iron, TSAT = transferrin saturation, UIBC = unsaturated iron-binding capacity, γ-GTP = γ-glutamyl transpeptidase. Obesity: body mass index ≥25 kg/m^2.

2.3. Statistical analysis

Data were analyzed using IBM SPSS Statistics 19 (IBM Corp., Chicago, IL). Dichotomous variables (sex, smoking status, and medications) were assigned values of 0 (female, non-smoker, and no) or 1 (male, smoker, and yes). Data with a normal distribution are expressed as means ± standard deviation, and data that were not normally distributed (BNP) are expressed as medians with interquartile ranges, evaluated after log transformation. Comparisons among multiple subgroups were made using 1-way analysis of variance followed by Scheffe post hoc test. Comparisons between continuous variables were performed using Pearson correlation (r). Multivariable regression analyses were performed as appropriate. Logistic regression analysis was performed to evaluate the associations of NTBI with the combination of higher levels of both d-ROM and BNP. A 2-tailed $P < .05$ was considered significant.

3. Results

A total of 1244 individuals—801 men and 443 women—were enrolled, of whom 243 were current smokers (19.5% of the total). Table 1 presents the characteristics of the population. Serum NTBI was detectable in all participants. Serum Fe, UIBC, NTBI, and d-ROM showed almost normal distributions, with median values of 105 μg/dL, 213 μg/dL, 0.20 μmol/L, and 349 Carratelli units, respectively (Fig. 1). Serum NTBI was higher in male than in female participants and decreased with age (Fig. 2). When participants were grouped into quartiles according to NTBI values, levels of Fe, UIBC, d-ROM, and BNP showed significant trends across quartiles of NTBI (Fig. 3).

Univariate regression analysis was performed to identify factors significantly related to NTBI concentration (Table 2). Subsequently, multivariable regression analysis was conducted to specify factors significantly associated with NTBI, including those correlated with NTBI in univariate analysis, as well as conventional cardiovascular risk factors such as smoking status, BP, lipid profiles, and glucose metabolism as independent variables. The multivariable regression analysis revealed positive correlations of LDL-C and Fe with NTBI concentration and inverse associations of NTBI concentrations with age, d-ROM, and BNP (Table 3). In the next series of multivariable analyses, we included NTBI as an independent variable to evaluate the relationships of NTBI with d-ROM or BNP. NTBI concentration was significantly associated with levels of d-ROM or BNP in the multivariable regression analyses after adjustment for possible confounding factors (Supplementary Tables 1, http://links.lww.com/MD/F662 and 2, http://links.lww.com/MD/F663).

We next performed logistic regression analysis to examine the associations of NTBI with the endpoint for the combination of levels above the upper quartiles for both d-ROM and BNP. The analysis revealed that NTBI was independently associated with the combination of higher levels of both d-ROM and BNP after adjustment for possible confounding factors (Table 4).

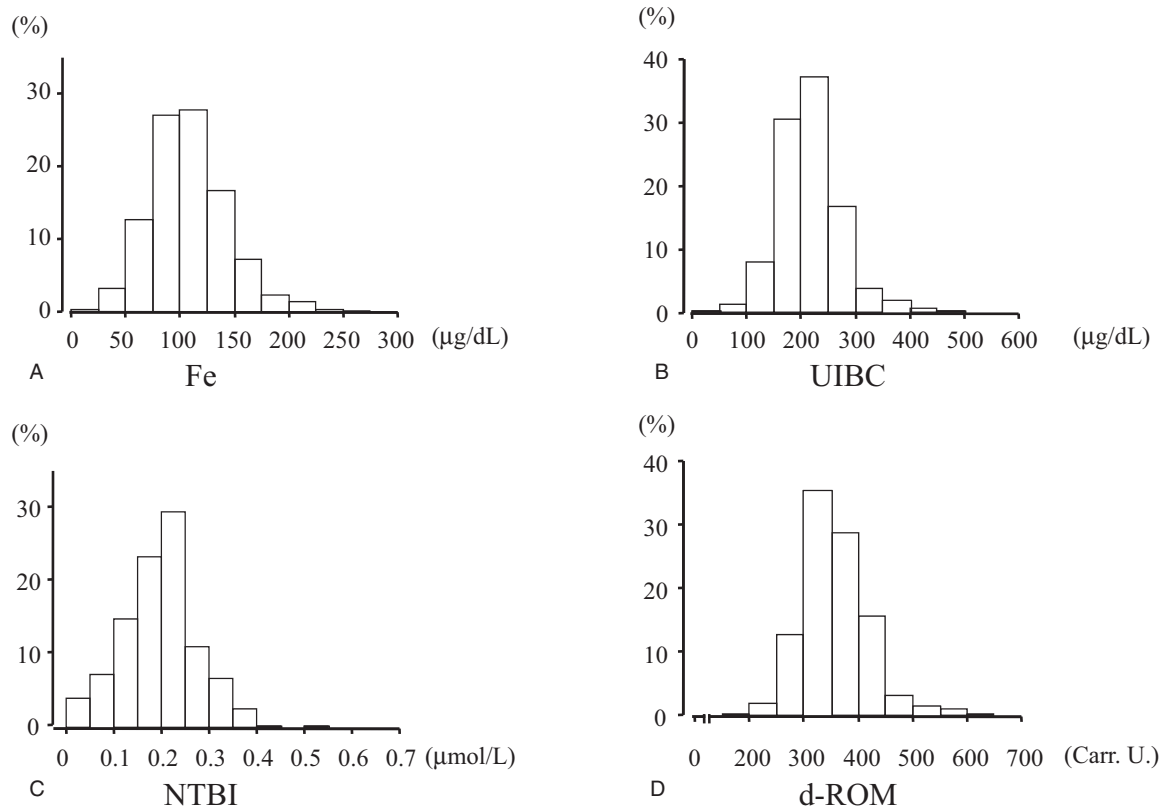

Figure 1. Distribution of (A) serum iron (Fe), (B) unsaturated iron-binding capacity (UIBC), (D) non–transferrin-bound iron (NTBI), and (D) derivatives of reactive oxygen metabolites (d-ROM). The d-ROM levels are expressed in Carratelli units (Carr. U.). The vertical axis indicates the percentage of the total participants.

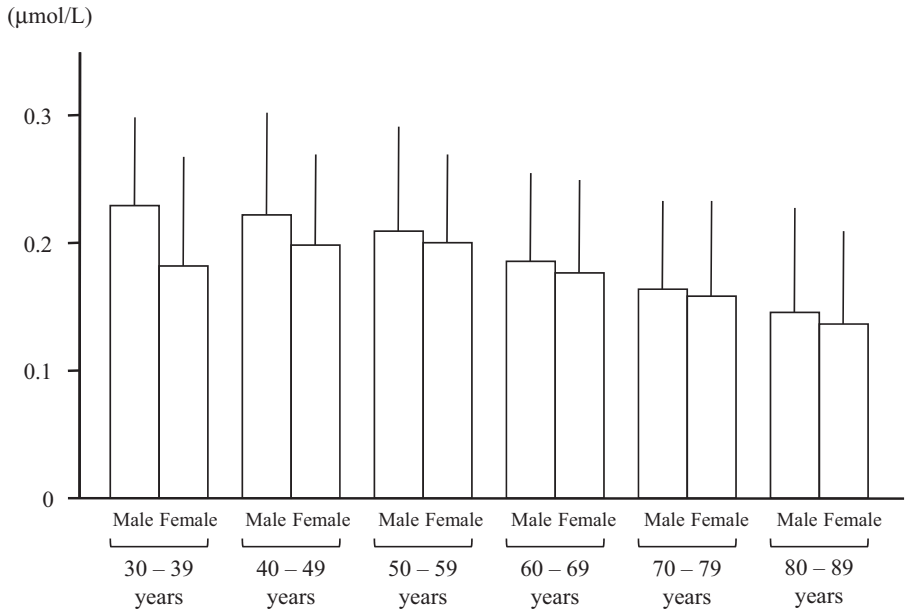

Figure 2. Distribution of non–transferrin-bound iron (NTBI) concentration based on age and gender.

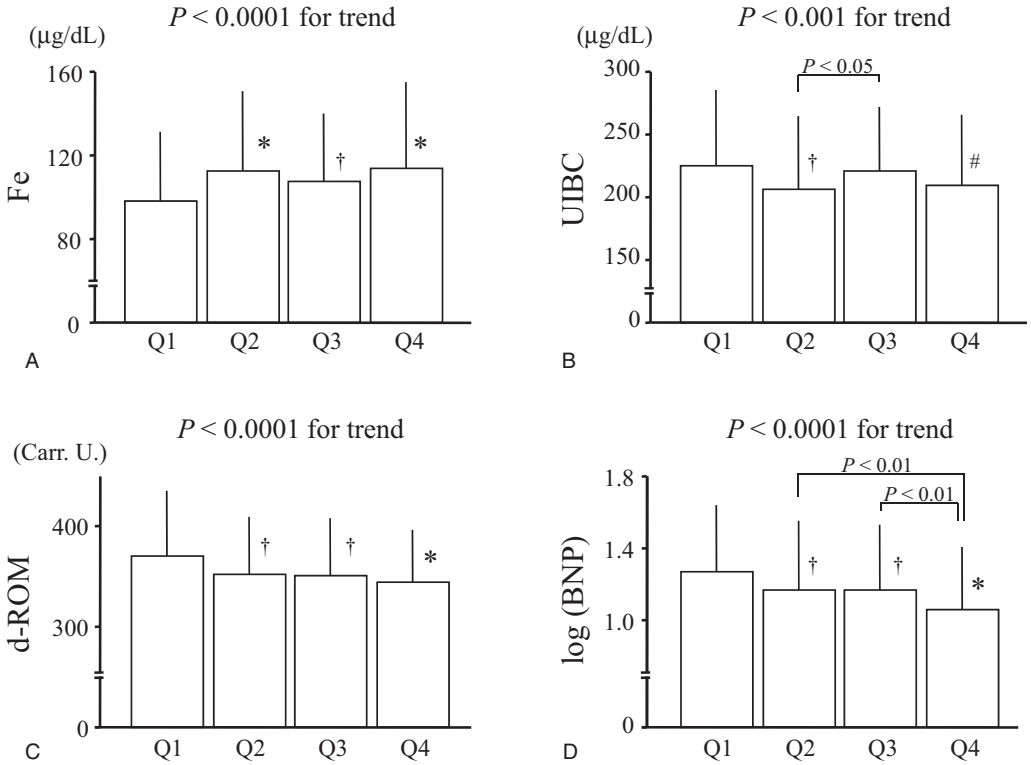

Figure 3. Relationship of (A) serum iron (Fe), (B) unsaturated iron-binding capacity (UIBC), (C) derivatives of reactive oxygen metabolites (d-ROM), and (D) B-type natriuretic peptide (BNP) with quartiles of non–transferrin-bound iron (NTBI). The d-ROM levels are expressed in Carratelli units (Carr. U.). The BNP values are log transformed. Q1 = NTBI values below the lower quartile, Q2 = NTIB values above the lower quartile and below the median, Q3 = NTIB values above the median and below the upper quartile, Q4 = NTBI values above the upper quartile. $^{*}P < .0001$, $^{\dagger}P < .01$, $^{\#}P < .05$ vs Q1 (one-way analysis of variance followed by Scheffe post hoc test).

Table 2

Results of possible correlattions between non–transferrin-bound iron (NTBI) and related factors in all participants ($n = 1244$).

Variable	Coefficient (r)	P
Age (years)	−0.261	<0.0001
Male sex (%)	0.084	<0.01
Body mass index (kg/m^2)	0.037	0.189
Current smoker (%)	0.082	<0.01
Systolic BP (mmHg)	−0.062	<0.05
Diastolic BP (mmHg)	0.018	0.528
Pulse rate (bpm)	0.005	0.867
Hemoglobin (g/dL)	0.112	<0.0001
AST (U/L)	−0.110	<0.001
ALT (U/L)	0.045	0.109
γ-GTP (U/L)	−0.012	0.676
Creatinine (mg/dL)	0.004	0.874
HDL-C (mg/dL)	−0.001	0.974
LDL-C (mg/dL)	0.072	<0.05
Triglycerides (mg/dL)	0.046	0.102
FPG (mg/dL)	−0.027	0.335
Fe (μg/dL)	0.157	<0.0001
UIBC (μg/dL)	−0.079	<0.01
TSAT (%)	0.134	<0.0001
Ferritin (ng/mL)	0.034	0.225
d-ROM (Carratelli units)	−0.161	<0.0001
BNP (pg/mL)	−0.198	<0.0001
Cardiovascular risk factors		
Hypertension (%)	−0.094	<0.001
Dyslipidemia (%)	0.054	0.055
Diabetes mellitus (%)	−0.059	<0.05
Obesity (%)	0.022	0.438
Medications		
ACEi or ARB (%)	−0.024	0.397
β-blocker (%)	−0.079	<0.01
Calcium channel blocker (%)	−0.098	<0.001
Diuretics (%)	−0.022	0.429
Lipid-lowering drug (%)	−0.046	0.102
Hypoglycemic drug (%)	−0.041	0.152
Antithrombotic agent (%)	−0.059	<0.05

ACEi = angiotensin-converting enzyme inhibitor, ALT = alanine transaminase, ARB = angiotensin receptor blocker, AST = aspartate transaminase, BNP = B-type natriuretic peptide, BP = blood pressure, d-ROM = derivatives of reactive oxygen metabolites, FPG = fasting plasma glucose, HDL-C = high-density lipoprotein cholesterol, LDL-C = low-density lipoprotein cholesterol, TSAT = transferrin saturation, UIBC = unsaturated iron-binding capacity, γ-GTP = γ-glutamyl transpeptidase. Obesity: body mass index ≥25 kg/m^2.

4. Discussion

The main findings of the present study are that

(1) serum NTBI could be detected at low levels in all participants;
(2) LDL-C and Fe were positively associated with NTBI, whereas age, d-ROM, and BNP were inversely associated; and
(3) NTBI was independently associated with the combination of higher levels of both d-ROM and BNP after adjustment for possible confounders. These findings indicate that serum NTBI at low levels might not have adverse effects and could play an as-yet-unknown physiological role, at least in seemingly healthy individuals without overt cardiovascular disease or iron overload.

To the best of our knowledge, this study is the first to investigate serum NTBI concentration in more than 1000 people without iron overload or pathological conditions. A few previous studies enrolled healthy individuals as controls and reported their NTBI concentrations, but these reports included only a limited number of participants (<100).[16–18] In the present study, serum NTBI was detectable in all participants although quite low. The mean value for NTBI was 0.193 ± 0.077 μmol/L, which was lower than the previously reported value (0.440 ± 0.076 μmol/L) measured using a similar analytical method.[16] Other studies have included patients with diabetes and reported higher NTBI concentrations in these patients than in controls.[17,18] However, diabetes was inversely correlated with NTBI concentration in the present study though it was the result in the univariate regression analysis. We do not know the reason for these inconsistencies, but speculate that differences in patient data, such as age, body mass index, and status of diabetes management, along with the study sample size may have been influences. Alternatively, NTBI concentration might vary within a low range, even in seemingly healthy individuals. Meanwhile, NTBI concentration in pathological conditions such as hematopoietic diseases or iron overload are reported at much higher concentrations, from 1.0 to 4.0 μmol/L, than in physiological conditions.[19–21]

As noted, NTBI is a labile free iron that increases ROS production and oxidative stress by catalyzing the Fenton and Haber–Weiss reactions.[1–3] Previous studies have shown that diabetic patients with elevated NTBI experience increased oxidative stress, based on measures of malondialdehyde.[17,18] Fe levels in the present study were significantly associated with NTBI concentration but were distributed in a unimodal peak, without excessively high levels; thus, most participants could not be considered as having an iron-overload condition. We also identified quite low NTBI concentrations compared to earlier reports describing patients with iron overload,[19–21] which is to be expected. Also in contrast to these studies, in the current work, oxidative stress as assessed using d-ROM was inversely associated with NTBI concentration. Causal relationships for this discrepancy are not evident but could reflect the difference between populations with known pathology and the presumably unaffected participants enrolled in our study. As noted, serum NTBI is linked to increased oxidative stress through induction of excessive ROS production in iron-overload states.[1–3] However, a low concentration of NTBI in physiological conditions without iron-overload may not have harmful effects. Other investigators have reported that d-ROM levels do not change significantly with increasing NTBI concentration after red blood cell transfusion,[22] but that study involved preterm infants and did not include assessment of other iron-related markers such as Fe and UIBC. The number of participants and their backgrounds also were quite different from ours, limiting comparison between those results and the current findings.

Levels of BNP, a neurohumoral factor reflecting cardiac load,[23,24] were normal or at most slightly elevated in the present study, consistent with what would be expected in seemingly healthy individuals. Moreover, BNP levels were inversely associated with NTBI concentrations in this population, similar to the results with d-ROM. In contrast, patients with heart failure often present with anemia complicated by iron use disorder, and their anemia cannot be ameliorated despite iron administration. These patients may well have elevated levels of both BNP and NTBI.[1,4,5] Of interest, we found an independent association of NTBI concentration with the combination of higher levels of both d-ROM and BNP. This result indicates a possible role for NTBI as an antioxidant in physiological conditions without iron or cardiac overload.

Table 3

Results of multivariable regression analysis showing factors possibly associated with non–transferrin-bound iron (NTBI) in all participants ($n = 1244$).

	Model 1		Model 2		Model 3		Model 4	
Variable	Standardized coefficient (β)	*P*	Standardized coefficient (β)	*P*	Standardized coefficient (β)	*P*	Standardized coefficient (β)	*P*
Age (years)	−0.261	<0.0001	−0.244	<0.0001	−0.193	<0.0001	−0.184	<0.0001
Male sex (%)	0.085	<0.01	0.117	<0.01	0.060	0.169	0.059	0.179
Body mass index (kg/m^2)	0.023	0.395	0.013	0.654	0.007	0.817	0.013	0.672
Current smoker (%)	–	–	−0.003	0.918	−0.008	0.795	−0.009	0.769
Systolic BP (mmHg)	–	–	−0.007	0.809	0.015	0.635	0.016	0.607
Hemoglobin (g/dL)	–	–	0.018	0.613	−0.027	0.479	−0.030	0.422
AST (U/L)	–	–	−0.045	0.118	−0.048	0.101	−0.048	0.100
Creatinine (mg/dL)	–	–	−0.045	0.199	−0.049	0.166	−0.044	0.213
LDL-C (mg/dL)	–	–	0.093	<0.01	0.079	<0.01	0.073	<0.05
FPG (mg/dL)	–	–	−0.009	0.754	−0.007	0.800	−0.003	0.924
Fe (μg/dL)	–	–	–	–	0.095	<0.05	0.103	<0.01
UIBC (μg/dL)	–	–	–	–	−0.008	0.840	−0.001	0.976
Ferritin (ng/mL)	–	–	–	–	0.013	0.692	0.019	0.554
d-ROM (Carratelli units)	–	–	–	–	−0.098	<0.01	−0.097	<0.01
BNP (pg/mL)	–	–	–	–	−0.081	<0.05	−0.076	<0.05
Medications								
β-blocker (%)	–	–	–	–	–	–	−0.044	0.121
Calcium channel blocker (%)	–	–	–	–	–	–	−0.021	0.483
Antithrombotic agent (%)	–	–	–	–	–	–	−0.008	0.786

AST = aspartate transaminase, BNP = B-type natriuretic peptide, B = blood pressure, d-ROM = derivatives of reactive oxygen metabolites, FPG = fasting plasma glucose, LDL-C = low-density lipoprotein cholesterol, UIBC = unsaturated iron-binding capacity.

Model 1 included age (years), male sex (%), and body mass index (kg/m^2). Model 2 included smoking status (yes or no), systolic BP (mmHg), hemoglobin (g/dL), AST (U/L), creatinine (mg/dL), LDL-C (mg/dL), and FPG (mg/dL) in addition to the factors in Model 1. Model 3 included Fe (μg/dL), UIBC (μg/dL), ferritin (ng/mL), d-ROM (Carratelli units) and BNP (pg/mL) and in addition to the factors in Model 2. Model 4 included the potential medications listed in this Table 1 in addition to the factors in Model 3.

On the other hand, we found a positive association between NTBI and LDL-C. In previous studies, a relationship of NTBI with LDL oxidation, but not LDL-C, was evaluated in patients with hemochromatosis, showing no significant association between them.[25,26] The relationship between NTBI and LDL-C, to the best of our knowledge, has not been investigated in the general population. High LDL-C levels are associated with metabolic disorders and induction of inflammation and oxidative stress.[27,28] Thus, low grade of inflammation caused by LDL-C might increase NTBI via oxidative stress-independent pathway. The association between NBTI and LDL-C in seemingly healthy population may be a clue for the understanding of pathophysiology of dyslipidemia and iron metabolism.

The current study participants were seemingly healthy individuals without iron overload, and NTBI accumulation thus was unlikely to be present in major organs such as the heart and liver. A healthy iron balance is controlled by hepcidin, a peptide synthesized in the liver. Through modulation of its receptor and cellular iron exporter ferroportin, hepcidin regulates intestinal iron absorption, plasma iron concentration, tissue iron distribution, and intracellular and extracellular distribution.[1,2,29,30] NTBI is also distributed in extracellular fluid, intracellular cytosol, tissues, and organs but is not entirely controlled by hepcidin and the ferroportin system.[1,2,29,30] Detailed mechanisms regarding NTBI metabolism have not been fully elucidated, but reports have described the elimination of excessive distributed NTBI in the presence of specific transporters.[31] We did not evaluate the intracellular and tissue distribution of NTBI, but our results may reflect part of the picture of NTBI metabolism under physiological conditions.

Table 4

Logistic regression analysis showing associations of non–transferrin-bound iron (NTBI) with the endpoint for the combination of levels above the upper quartiles for both derivatives of reactive oxygen metabolites (d-ROM) and B-type natriuretic peptide (BNP) in all participants ($n = 1244$).

	Model 1		Model 2		Model 3		Model 4	
Variable	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
NTBI (per 0.1 μmol/L)	0.652 (0.467–0.910)	<0.05	0.696 (0.494–0.980)	<0.05	0.703 (0.498–0.992)	<0.05	0.697 (0.491–0.989)	<0.05

CI = confidence interval, NTBI = non–transferrin-bound iron.

The endpoint of logistic regression analysis was a combination of levels above the upper quartile for both d-ROM (>391 Carratelli units) and BNP (>27.9 pg/mL).

Model 1 was adjusted for age (years), male sex (%), and body mass index (kg/m^2). Model 2 was adjusted for smoking status (yes or no), systolic blood pressure (mmHg), pulse rate (bpm), hemoglobin (g/dL), aspartate transaminase (U/L), creatinine (mg/dL), high-density lipoprotein cholesterol (mg/dL), low-density lipoprotein cholesterol (mg/dL), triglycerides (mg/dL), and fasting plasma glucose (mg/dL) in addition to the factors in Model 1. Model 3 was adjusted for Fe (μg/dL), unsaturated iron-binding capacity (μg/dL), and ferritin (ng/mL) in addition to the factors in Model 2. Model 4 was adjusted for the potential medications listed in Table 1 in addition to the factors in Model 3.

5. Limitations

The present study has several limitations, and the findings should thus be interpreted with caution. First, the design was cross-sectional, and the study participants were a heterogeneous group. Second, we did not capture causal relationships among NTBI, d-ROM, and BNP, and *in vivo* studies should clarify the underlying mechanisms. Third, study participants were seemingly healthy individuals enrolled from the general population but might not be free of hidden hematopoietic diseases or disorders of iron metabolism. Fourth, we did not evaluate iron accumulation in tissues or organs or intracellular iron metabolism. Fifth, individuals taking iron supplementation as dietary supplements could not be excluded, but serum levels of Fe or ferritin were within normal limits in most participants and no participant showed higher levels than twice the normal upper limits in the present study. For definitive conclusions, further studies are needed that include a larger population, a longitudinal design, and detailed clinical examinations.

6. Conclusions

Serum NTBI was detected at low levels in the general population, and significant inverse associations of NTBI with d-ROM and BNP were revealed. These findings indicate that serum NTBI in physiological conditions does not necessarily reflect increased oxidative stress, in contrast to the implications of higher levels in states of iron overload or pathological conditions.

Author contributions

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Funding acquisition: Satoshi Fujii, Nobuyuki Ohte.
Investigation: Tomonori Sugiura, Satoshi Fujii.
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Visualization: Hiroyuki Takase.
Writing – original draft: Tomonori Sugiura, Yasuaki Dohi.
Writing – review & editing: Yasuaki Dohi, Hiroyuki Takase, Satoshi Fujii, Yoshihiro Seo, Nobuyuki Ohte.

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