Improvement in the Diagnostics of Iron Deficiency Anemia using Multiple Marker Combinations
(Penambahbaikan dalam Diagnostik Anemia Kekurangan Zat Besi menggunakan Gabungan Penanda Berganda)

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ABSTRACT

Anemia of chronic disease (ACD) frequently occurred in patients with chronic inflammatory diseases and can be treated by treating the underlying disease. On the other hand, iron-deficiency anemia (IDA), the most common type of anemia, occurred with iron loss or when the iron requirement of the body was increased. Since the treatment methods for ACD and IDA differ, it is important to clinically distinguish between the two types of anemia. In this study, we investigated and evaluated the performance of a number of biomarkers, including ferritin, soluble transferrin receptor (sTfR), hepcidin, C-reactive protein (CRP) and combination markers containing ferritin for the diagnosis of IDA using serum samples from Korean patients (80 ACD and 48 IDA Korean patients). Among the single markers, ferritin exhibited the best performance with 98.58% AUC and 97.50% sensitivity. In this study, a combination of two biomarkers was used to differentially diagnose IDA and ACD. Among the combination markers, ferritin + sTfR showed the best performance with 99.51% AUC and 98.75% sensitivity. We found that the ferritin + sTfR combination showed the best diagnostic performance with 1.25% higher SN than ferritin alone. Moreover, it also showed 10% better diagnostic performance than the single ferritin marker within the data range where the distinction between ACD and IDA is unclear. We propose that using combination markers containing ferritin may diagnose IDA more accurately and facilitate the determination of the appropriate anemia treatment to expedite patient recovery.

Keywords: Index; iron deficiency anemia; multianalyte; transferin serum

ABSTRAK

Anemia penyakit kronik (ACD) kerap berlaku kepada pesakit dengan penyakit radang kronik dan boleh dirawat dengan merawat penyakit yang mendasarinya. Sebaliknya, anemia kekurangan zat besi (IDA) adalah jenis anemia yang paling biasa berlaku dengan kehilangan zat besi atau apabila keperluan zat besi dalam badan bertambah. Oleh kerana kaedah rawatan untuk ACD dan IDA berbeza, adalah penting secara klinikal untuk membezakan antara kedua-dua jenis anemia. Dalam kajian ini, kami mengkaji dan menilai prestasi beberapa penanda biologi, termasuk feritin, transferin larut reseptor (sTfR), hepcidin, protein C-reaktif (CRP) dan penanda gabungan yang mengandungi feritin untuk diagnosis IDA menggunakan sampel serum daripada pesakit warga Korea (80 ACD dan 48 IDA pesakit warga Korea). Antara penanda tunggal, feritin menunjukkan prestasi yang terbaik dengan 98.58% AUC dan 97.50% kepekaan. Dalam kajian ini, gabungan dua penanda biologi telah digunakan untuk mendiagnosis IDA dan ACD secara berbeza. Antara penanda kombinasi, feritin + sTfR menunjukkan prestasi yang terbaik dengan 99.51% AUC dan 98.75% kepekaan. Kami mendapati bahawa gabungan feritin + sTfR menunjukkan prestasi diagnostik yang terbaik dengan 1.25% SN lebih tinggi daripada feritin sahaja. Selain itu, ia juga menunjukkan prestasi diagnostik 10% lebih baik daripada penanda feritin tunggal dalam julat data dengan perbezaan antara ACD dan IDA adalah tidak jelas. Kami mencadangkan bahawa penggunaan penanda gabungan yang mengandungi feritin boleh mendiagnosis IDA dengan lebih tepat dan memudahkan penentuan rawatan anemia yang sesuai untuk mempercepatkan pemulihan pesakit.

Kata kunci: Anemia kekurangan zat besi; indeks; pelbagai analit; serum transferin

INTRODUCTION

Anemia, a commonly occurring disease, is associated with low levels of red blood cells or hemoglobin and is known to affect one in 24 Americans. It is caused by the decreased production of red blood cells or hemoglobin, damage or destruction of red blood cells, chronic diseases and malignant tumors (MedicineNet.com). Anemia of chronic disease (ACD) frequently occurred in patients with chronic inflammatory diseases and can be treated by treating the underlying disease. On the other hand, iron-deficiency anemia (IDA), the most common type of anemia, occurred with iron loss or when the iron requirement of the body was increased. In the developed world, IDA shows a prevalence of 2-5% among adult men and post-menopausal

women, constitutes a common cause of referral to gastroenterologists (Calvey & Castleden 1987; Mclntyre & Long 1993). IDA has various physiological or pathological causes, which are important criteria for determining the precise treatment regimen of anemia (Clark 2009). For instance, blood loss from the gastrointestinal (GI) tract or menstrual blood loss commonly cause IDA in adult men and post-menopausal or in pre-menopausal women, respectively (Calvey & Castleden 1987; Mclntyre & Long 1993).

Iron is an essential nutrient for physiological processes such as oxygen transportation and cellular respiration. It is an important co-factor for various proteins such as hemoglobin, myoglobin and in heme complexes such as cytochromes. Iron deficiency can not only cause anemia, but may also affect nerve function and intelligence; thus, it is a serious disorder, especially in infants. Conversely, iron overload can cause problems such as hemochromatosis, hepatocirrhosis, diabetes, cardiomyopathy, arthritis and skin disease. Both iron deficiency and overload can be prevented by testing for the iron levels. While dietary iron supplementation may be helpful for IDA patients, it can prove harmful for ACD patients.

Since the treatment methods for ACD and IDA differ, it is important to clinically distinguish between the two types of anemia; however, making this distinction is challenging due to the low levels of serum iron and transferrin saturation exhibited by both ACD and IDA (Theurl et al. 2009). Especially in hospital, iron deficiency is clinically associated with inflammation and the typical iron pattern of IDA and ACD appears at the same time (Remacha et al. 1998).

The current gold standard for the diagnosis of IDA is bone marrow examination. Due to the invasive and costly nature of this method, efforts to develop novel, simpler, non-invasive *in vitro* diagnostic tests for IDA are ongoing (Chang et al. 2007; Geerts et al. 2012; Goyal et al. 2008; Lipschitz et al. 1974; Punnonen et al. 1997; Weiss & Goodnough 2005).

Ferritin is a biomarker commonly used to differentially diagnose IDA and ACD. Ferritin, an iron-storing protein is used as an indirect indicator of the total iron concentration in the body. IDA is diagnosed when the ferritin levels are <30 ng/mL, whereas in ACD, the ferritin levels are >100 ng/mL. However, patients with infection, inflammatory responses, or malignant tumors may also exhibit high levels of ferritin, making it difficult to diagnose IDA using ferritin alone (Lipschitz et al. 1974; Weiss & Goodnough 2005). Thus, numerous efforts have been undertaken to develop methods for differentiating between and diagnosing IDA and ACD using various combinations of inflammation-related serum proteins.

Soluble transferrin receptor (sTfR) is an indicator of the intracellular iron levels. Similar to ferritin, it is not affected by chronic diseases and has been reported to be a useful indicator of stored iron levels (MedicineNet.com; Chang et al. 2007; Geerts et al. 2012). C-reactive protein (CRP), an inflammatory response protein is used to identify patients with infections, inflammatory responses or malignant tumors, whereas the peptide hormone hepcidin is a regulator of iron homeostasis (Geerts et al. 2012).

In this study, we combined ferritin with the complementary biomarkers in an attempt to establish an accurate diagnostic method of IDA.

MATERIALS AND METHODS

STUDY POPULATIONS AND SAMPLE COLLECTION

The serum samples used in this study were obtained from 128 Korean anemic patients from the Hallym University Medical Center. The patient groups comprised of 80 ACD patients and 48 IDA patients.

IMMUNOASSAYS

Protein levels were measured using xMAP bead-based technology (Luminex, Austin, TX). The sera obtained from the anemic patients were incubated with Luminex-beads covered with ferritin, sTfR, hepcidin and CRP. The fluorescence levels of the antibodies from each bead were measured and calculated using standard curves created by Bio-Rad (five-parameter curve fitting). The Luminex xMAP technology is a multiplexed immunoassay measurement method that examines multiple biomarkers simultaneously making it superior in terms of cost, time and amount of samples processed, relative to other individual examination methods.

STATISTICAL ANALYSIS

Statistical analyses and performance measurements of the biomarkers were performed using the in-house-developed multi-biomarker analytical system (Cheong et al. 2013). The model used to differentiate between IDA and ACD must have the ability to test for both, the sensitivity (SN) and specificity (SP). A common method to test for adequately high SN and SP values is to calculate the area under the receiver operating characteristic curve (ROC-AUC) (Pepe et al. 2006). For performance measurements, AUC, SN and SP were determined by leave-one-out validations using a logistic regression classification algorithm.

RESULTS AND DISCUSSION

Figure 1 shows the box plots of IDA and ACD distributions for the individual biomarkers. In the ACD and IDA groups, the median ferritin concentrations are 111 μg/mL (mean±SD, 111±54.95 μg/mL) and 7 μg/mL (11.38±10.13 μg/mL); median sTfR concentrations are 11.65 μg/mL (11.67±4.78 μg/mL) and 32.36 μg/mL (38.4±23.28 μg/mL); median CRP concentrations are 19.35 pg/mL (38.92±56.71 pg/mL) and 3.18 pg/mL (14.61±26.88 pg/mL); and median hepcidin concentrations are 5.92 μg/mL (10.52±11.6 μg/mL) and 1.25 μg/mL (2.31±3.24 μg/mL), respectively. In the case of ferritin, ACD patients exhibit a wide range
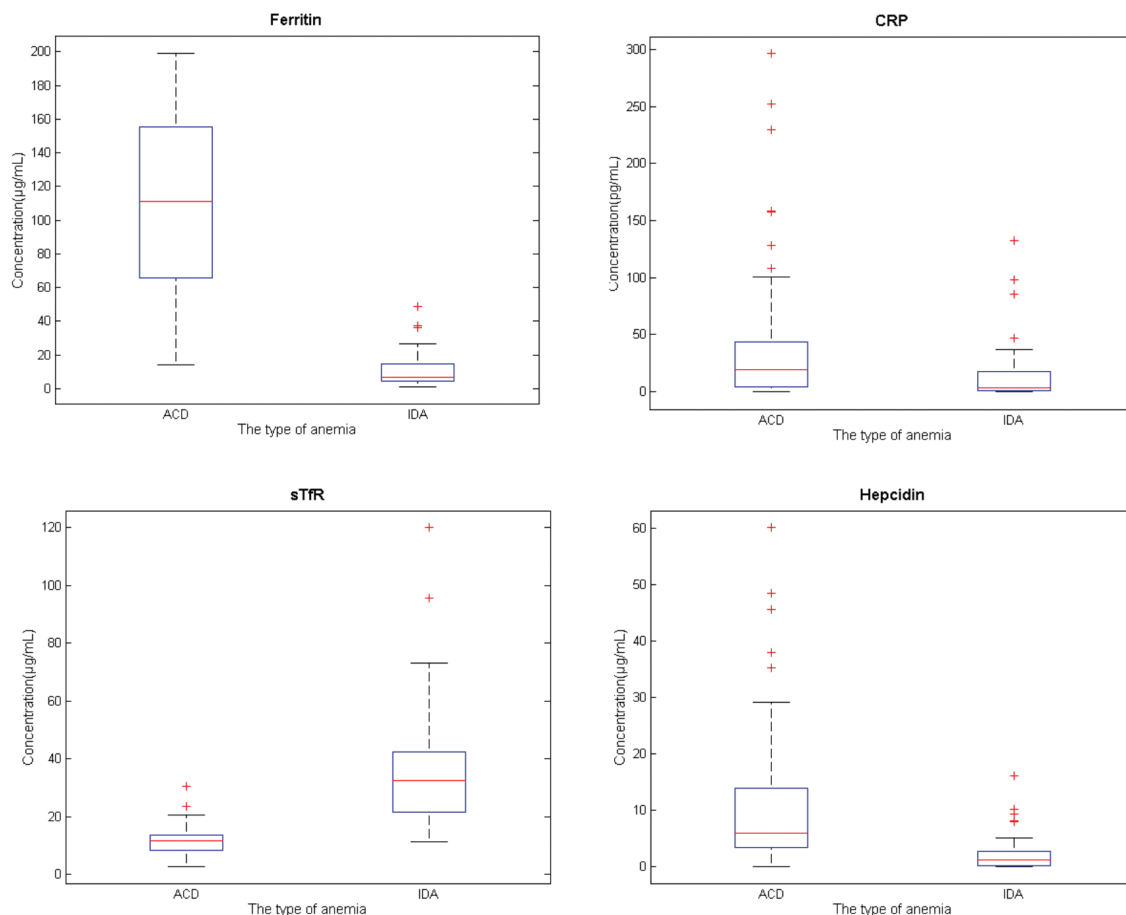
FIGURE 1. Box plots of the individual markers of ACD, anemia of chronic disease; IDA, iron deficiency anemia; sTfR, soluble transferrin receptor; and CRP, C-reactive protein

of distribution from 20-200 μg/mL, whereas IDA patients exhibit a relatively narrow distribution. ACD and IDA patients exhibit significantly distinct levels of ferritin and sTfR. In the case of CRP and hepcidin, differentiation of the median levels is negligible between the ACD and IDA patients.

Figure 2 depicts two-dimensional (2D) scatter plots of ferritin and other markers and clearly demonstrates the marker distribution for ACD and IDA. Figure 2(a), a scatter plot of ferritin and sTfR, shows that classification of ACD and IDA is more clear than scatter plots of ferritin/CRP (Figure 2(b)) and ferritin/hepcidin (Figure 2(c)).

Figure 3 shows the ROC curves for the single markers used to test for IDA diagnostic performance. Table 1 shows the calculated values of area under the receiver operating characteristic curve (ROC-AUC) and sensitivity (SN) using a 90% cutoff-point for specificity (SP). Among the single

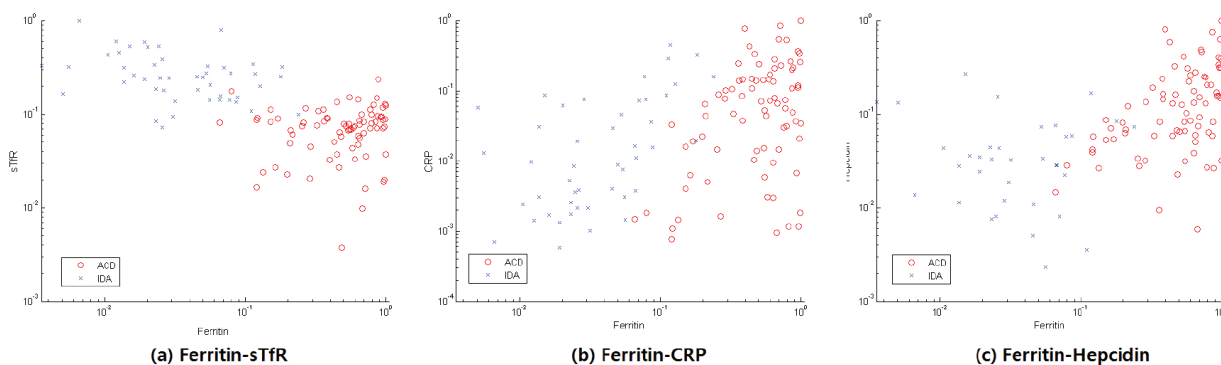**(a) Ferritin-sTfR**   **(b) Ferritin-CRP**   **(c) Ferritin-Hepcidin**

FIGURE 2. 2D scatter plots of ferritin combination markers (a) Ferritin and soluble transferrin receptor (sTfR), (b) ferritin and C-reactive protein (CRP) and (c) ferritin and hepcidin
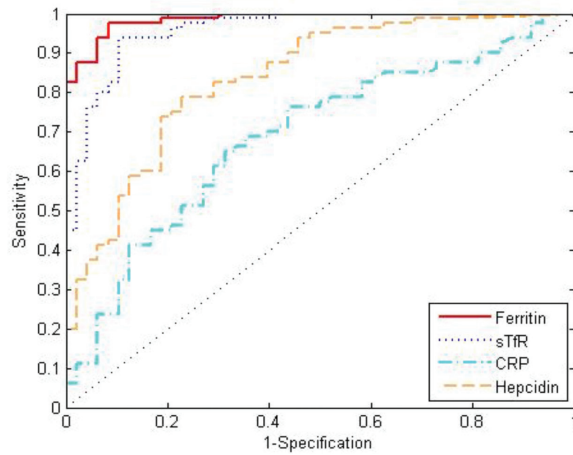
FIGURE 3. ROC curves for ferritin, soluble transferrin receptor (sTfR), hepcidin and C-reactive protein (CRP)

TABLE 1. ROC-AUC comparison of the single markers of anemia

Marker	AUC	95% CI	SN at 90% SP
Ferritin	98.58	96.24~99.58	97.50
sTfR	95.78	98.31~91.22	81.25
Hepcidin	83.72	73.88~89.91	42.50
CRP	68.76	59.00~77.41	23.75

markers, ferritin exhibited the best performance with 98.58% AUC and 97.50% SN. sTfR showed a weaker performance than ferritin with 95.78% AUC and 81.25% SN. AUC and SN values for hepcidin and CRP were 83.72 and 42.50% and 68.76 and 23.75%, respectively, indicating that they are relatively poor diagnostic markers for anemia. Since these markers provide information regarding inflammation, they can be used as combination markers with anemia-related markers.

In this study, a combination of two biomarkers was used to differentially diagnose IDA and ACD. Ferritin, the most commonly used marker for anemia, shows the best performance in differentiating between IDA and ACD. In this study, ferritin was combined with the other markers. Figure 4 shows the ROC curves of these combination markers and Table 2 shows their ROC-AUC and SN values.

Among the combination markers, ferritin + sTfR showed the best performance with 99.51% AUC and 98.75% SN. Ferritin + CRP showed 99.22% AUC and 97.50% SN, whereas ferritin + hepcidin showed 98.49% AUC and 95.00% SN. Ferritin + sTfR and ferritin + CRP exhibited similar 95% confidence intervals (CI) of 97.83-100.0 and 97.30-99.83, respectively, indicating a strong diagnostic performance of both of these combinations.
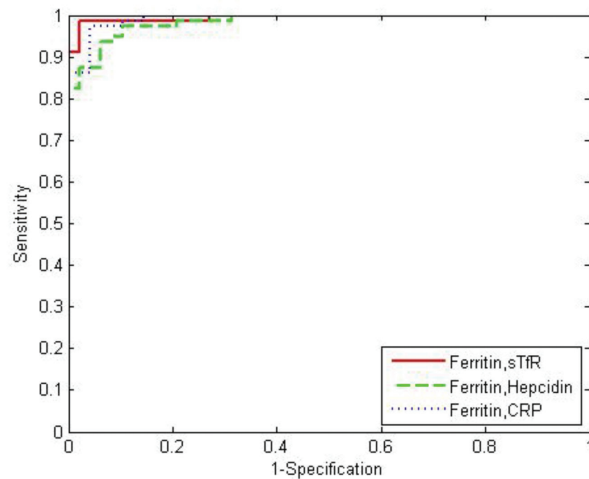

FIGURE 4. ROC curves for combination markers of anemia

TABLE 2. ROC-AUC comparison of combination markers of anemia

Marker	AUC	95% CI	SN at 90% SP
Ferritin+sTfR	99.51	97.83-100.0	98.75
Ferritin+CRP	99.22	97.30-99.83	97.50
Ferritin+Hepcidin	98.49	96.28-99.50	95.00

Due to the difficulty in differentiating between ACD and IDA with ferritin values of 10-100 ng/mL, the data within this range were sampled and compared for diagnostic performance.

Figure 5 shows the ROC curves for ferritin as a single marker and combination markers containing ferritin, between the ferritin values of 10-100 ng/mL. Table 3 compares their ROC-AUC and SN performance.

Within the data range, the ferritin single marker had an ROC-AUC value of 94.49% and SN of 77.78%. In addition, the ferritin + sTfR combination showed the best performance with 99.48% AUC and 97.78% SN, which is similar to its performance in the overall data. Also, ferritin + CRP and ferritin + hepcidin showed 96.97 and 94.04% AUC and 95.55 and 77.78% SN, respectively, which indicates a weaker performance, compared to their overall data.

The ROC curve in Figure 6(a) shows that ferritin and ferritin + sTfR exhibit the best performance among the single and combination markers, respectively. The ROC curve in Figure 6(b) exhibits the performance of limited ferritin data between 10-100 ng/mL. Ferritin and ferritin + sTfR exhibit 98.58 and 99.51% AUC and 97.5 and 98.75%

sensitivity, respectively. In other words, the performance of the combination markers was increased by 1% compared to the ferritin marker alone. The combination of ferritin and sTfR shows the highest AUC and 95% CI for both the single and combination markers. Limited Ferritin combination from 10-100 ng/mL has 98.05% on AUC and 97.14% on sensitivity. Although the sample size of this data was smaller than the unlimited data, ferritin as a single marker has only 1.5% difference with the combination of ferritin and sTfR.

CONCLUSION

In this study, the performance of ferritin, sTfR, hepcidin and CRP as single markers and combination markers containing ferritin, was tested to determine the most accurate diagnostic method for IDA. We found that the ferritin + sTfR combination showed the best diagnostic performance with 1.25% higher SN than ferritin alone. Moreover, it also showed 10% better diagnostic performance than the single ferritin marker within the data range where the distinction between ACD and IDA is unclear. The physiological and pathological causes of IDA are diverse and they become
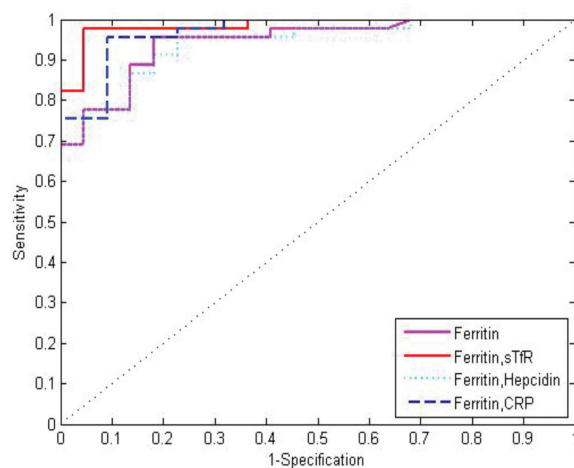

FIGURE 5. ROC curves of ferritin data range (10-100 ng/mL)

TABLE 3. ROC-AUC comparison of combination markers for ferritin data range (10-100 ng/mL)

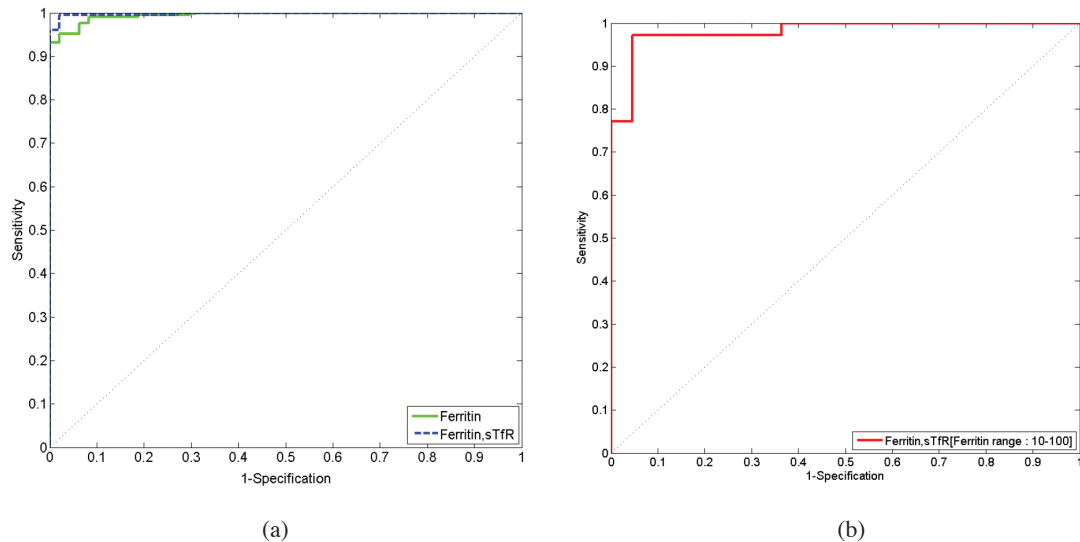| Marker	AUC	95% CI	SN at 90% SP
Ferritin	94.49	86.77-98.14	77.78
Ferritin+ sTfR	98.48	94.03-100.0	97.78
Ferritin+CRP	96.97	90.95-99.49	95.56
Ferritin+Hepcidin	94.04	86.15-97.63	77.78

FIGURE 6. ROC curves of anemia markers showing the best performance with (a) all data and (b) Ferritin data range

important evaluation criteria when selecting the most appropriate treatment regimen for anemia. Therefore, using combination markers containing ferritin and other markers related to the diverse causes of IDA, may diagnose IDA more accurately and facilitate the determination of the appropriate anemia treatment to expedite patient recovery.

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