 doi:10.20944/preprints201912.0356.v1

Evaluation of erythroferrone, hepcidin, and iron overload status in Iraqi transfusion-dependent β-Thalassemia major patients

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 doi:10.20944/preprints201912.0356.v1

Abstract

Beta thalassemia major (β-TM) disorder characterized by the lack, or severe reduction in the production of hemoglobin β-globin chains. The standard protocol for the management of β-TM is blood transfusion and iron chelation therapy to reduce the iron overload state. The present study aimed to investigate the relationships between two iron regulatory hormones, hepcidin (HEPC) and erythroferrone (ERFE) levels and iron status parameters (ISPs) in Iraqi patients with β-TM. ISPs and hormones were measured in sixty patients and compared with thirty healthy controls. The results indicated significant changes in different iron status parameters, while ferritin (FRT) with the ~11 fold increase showed the most change. Significant reduction in HEPC and increase in ERFE levels were detected in patients as compared to the control group, while no direct correlation was identified with the other measured ISPs. Receiver operating characteristic (ROC) analysis showed that the z-score of the composite of ERFE+FRT has a full diagnostic ability for β-TM. In conclusion, our finding indicated the correlation between different ISPs, FRT as the leading predictor of iron overload and tow main iron regulatory hormones.

Keywords: Beta-thalassemia, Erythroferrone, Ferritin, Hepcidin, Iron overload.

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Introduction

β-TM is a hereditary disease characterized by the absence or high reduction in the production of β-globin chains that leads to an increase in the α/β globin ration. In patients with β-TM, excess free α-globin chains aggregate and precipitate in erythroblast (Heinz bodies) leading to disruption of the cell membrane and generation of reactive oxygen species (ROS) and subsequently ineffective erythropoiesis [1]. Hence, β-TM is characterized by microcytic anemia, ineffective erythropoiesis with increased concentration of erythropoietin which shortens red cell survival, and associates with cumulative iron overload [2]. There may be a range of clinical symptoms, including severe anemia, growth retardation, poor musculature, fatigue, and hepatosplenomegaly that lead to shortened life expectancy if left untreated [3].

The current therapies for β-TM include blood transfusion yielding almost normal life expectations [4, 5]. Since the human body has no regulated mechanism to excrete excess iron, secondary iron overload occurs rapidly in patients, who are on chronic blood transfusion programs. Even in non-transfused patients, iron overload improves slowly due to ineffective erythropoiesis [6].

Iron overload is the major cause of organ injury and mortality in β-TM; hence, patients with this condition rely on iron chelation therapy to remove excess iron [7, 8].

HEPC, a small peptide hormone secreted by hepatocyte, and erythroferron that inhibit the action of HEPC, are among the central regulators of iron homeostasis in human plasma and promising therapeutic targets for iron disorders. HEPC inhibits iron influx into plasma by regulating the cellular concentration of the sole known cellular iron exporter called ferroportin [9, 10]. It was found that HEPC binds to the central cavity of ferroportin in the surface of enterocytes, macrophages, and hepatocytes, and blocks its iron export activity. Furthermore, upon HEPC binding,

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conformational changes take place in ferroportin that associate with the exposure of its ubiquitination sites and therefore initiation of internalisation and degradation [11]. Malfunction of the HEPC-ferroportin axis underlies some common iron disorders, such as iron overload in β-TM, anemia of inflammation, or cancer [12].

HEPC deficiency, which is the consequence of ineffective erythropoiesis, is one of the primary factors that induce iron overload in iron-loading anemia such as β-TM [13]. The blood transfusion procedure may slightly ameliorates ineffective erythropoiesis and increases HEPC level but will lead to an iron overload on the other side [6, 14]. Since of association between HEPC dysregulation and iron overload diseases, fine-tuning of HEPC concentration in the body would be suggested as an efficient strategy to alleviate the disease's symptoms [15].

ERFE is a protein hormone produced by erythroblasts and mediates suppression of HEPC expression in the liver [16, 17]. The high increment of ERFE in a mouse model of thalassemia intermedia, and restoration of normal levels of HEPC after its ablation, suggesting ERFE could be a reason for HEPC production drop in β-TM [18]. Thus and so ERFE is another therapeutic candidate for the treatment of HEPC mediated anemia such as anemia of inflammation or iron-refractory iron-deficiency anemia [19].

In this study, the correlation between HEPC, ferroportin, and ERFE levels with ISPs was compared in Iraqi β-TM patients and healthy control groups.

Materials and Methods

Subjects

The present study included sixty β-TM patients who have received a regular blood transfusion at the Thalassemia Unit, "Al-Zahra'a Teaching Hospital", Najaf, Iraq. Also, a total of thirty healthy

 doi:10.20944/preprints201912.0356.v1

Iraqi subjects, without a history of anemia or hematological disorders, were used as the control population. The subject ages ranged from 1.9 to 8.7 years, with a median value of 5.9.

Diagnosis of β-TM was made by hematologists according to the 2019 edition of ICD-10-CM D56.1. The percentage of HbA2 and HbF were detected using HPLC (Variant II Beta Thalassemia Short Program, Bio-Rad Laboratories Inc., USA) according to conditions specified in the manual of the manufacturer. The values of 1.7-3.25% and < 1% were considered as the normal range for HbA2 and HbF, respectively.

All β-TM patients were on a regular blood transfusion regimen (15 ml of packed RBCs/kg of body weight every 2-6 week) to maintain the post-transfusion Hb above 9.5 g/dl. All patients received iron chelation therapy by subcutaneous Desferal (desferrioxamine B) infusion in a dose of 30 to 60 mg/kg body weight/day, 4 days/week. None of the patients underwent splenectomy, and serum C-reactive protein (CRP) test was used to exclude those participants with CRP higher than 6 mg/ml as these levels are clinically indicative of inflammation or infection that may distort the results [20].

Different medical tests, including endocrine, cardiac and systemic disorder evaluations were performed at the time of sample collection, and only subjects with normal results were enrolled in the study. Blood samples from the patient group were collected at least 7-10 days after or before the blood transfusion.

The study was approved by the local ethics committee of the University of Kufa (REC number: 472/2018) and written informed consents of all participants were obtained prior to the study.

Sample preparation

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Approximately 5 ml of participants' blood was collected using standard venipuncture technique. Half of each sample was transferred into sterile tubes with anticoagulant ethylenediaminetetraacetic acid (EDTA) for hematological analysis. For serological and biochemical analysis, the rest of the specimens were transferred into plain tubes to extract the serum. The clots were removed from the latter samples by centrifugation at 3000 rpm for 10 min and the resulting supernatant, designated serum, stored at -80 °C until analysis.

Quantification of serum markers for iron status

Serum ERFE and HEPC levels were measured using enzyme-linked immunosorbent assays (ELISA) using human ERFE ELISA kit (Mybiosourse, MBS2088219), and human HEPC Duoset ELISA kit (R&D systems, DY8307), respectively. Serum FRT concentration level was detected using VIDAS® FRT quantitative test with enzyme-linked fluorescent assay (ELFA) technique. Serum iron and total iron-binding capacity (TIBC) (µM) were measured biochemically using colourimetric assay by commercial kits (BioLab). The other ISPs including unsaturated iron-binding capacity (UIBC), transferrin saturation percentage (TS%), and transferrin concentration were calculated according to previously described formula [20, 21].

Statistical Analysis

The t-test was used to compare observation in two groups (β-TM *vs* control), and χ^2-test was used to compare the nominal variables between two groups (*e.g.* gender and diagnosis). The nonlinearity of both the mean and the variance of any biomarker is a predictable source of variance that is eliminated through the use of *z*-scores. We computed four z unit weighted composite scores

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based on the iron regulatory proteins levels (zERFE-zHEPC, zERFE+zFRT, and zERFE-zHEPC+zFRT).

Correlation analysis between variables was calculated using Pearson's and Spearman's correlation coefficients.

Multivariate general linear model (GLM) analysis was used to delineate the effects of diagnosis on the biomarkers and their composite score while controlling for confounding variables, including gender and age. Subsequently, multiple protected comparisons among groups means and tests for between-subject effects, to evaluate the effects of independent variables on biomarkers, were performed. Model-generated estimated marginal mean (± SE) values were computed and expressed as z-score.

The binary logistic regression analysis was used to check the predictors of β-TM (dependent variable) versus controls, including odds ratios with 95% confidence intervals. Multiple regression analyses were employed to assess the most significant biomarkers that predict β-TM disorder. The ROC plot was generated by plotting the sensivity versus 1-specifity in order to compare the predictive efficiency of potential biomarkers (and their combination) as well as the area under curve (AUC). The Youden's J statistics was used to determine cut-off scores, and alpha was set at 0.05.

All statistical analyses were performed using IBM SPSS windows version 25, 2017, using a 2-tailed test at α=0.05 and assuming an effect size of 0.13 with a power of 0.80. The number of subjects was 90, the tests were 2-tailed and a *p*-value of <0.05 was considered to indicate statistical significance.

Results and discussion

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Clinical data in β-TM and control groups

The socio-demographic data and raw values, collected using questionnaire, were shown in table 1. There were no significant differences in age and sex between β-TM and control groups. Significant differences (p<0.05) in all iron indices and iron regulatory hormones were observed in patient and control groups.

Table 1. Socio-demographic and clinical data (expressed as mean ± SD or median) in β-TM

Parameters	Patients N=60	Control N=30	p-value
Age (years)	8.71±2.59	7.95±3.25	0.071
Sex (M/F)	27/33	13/17	0.267
Iron (µM)	44.30±10.56	18.17±3.84	<0.001
TIBC (µM)	64.88±10.66	54.25±4.78	0.013
UIBC (µM)	20.58±10.28	36.08±6.45	<0.001
TS%	68.53±14.24	33.82±8.08	<0.001
Transferrin (mg/L)	163.01±22.44	136.31±12.01	<0.001
FRT (ng/mL)	1224(915-1381)	111.54(81-180)	<0.001
HEPC (ng/mL)	36.06(23.00-80.45)	80.34(31.62-110.72)	0.005
ERFE (ng/mL)	78.83(51.26-156.38)	25.11(14.18-41.22)	<0.001

Data analysis and biomarker differences between β-TM and healthy controls

Table 2 shows the outcome of a multivariate GLM analysis with all biomarkers as dependent variables while adjusting for sex and age. The dependent variables were ISPs, ERFE, HEPC, and FRT as well as complex weighted parameters including zERFE-zHEPC, zERFE+zFRT and zERFE-zHEPC+zFRT. We found a significant effect of diagnosis with an effect size of 0.533, while age, sex, number of transfusion and duration of disease were not significant. There were highly significant associations between all the biomarkers and diagnosis with the strongest associations between diagnosis and iron with effect size = 0.541, followed by TS% and FRT with effect sizes of 0.527 and 0.510, respectively. The composite zERFE+zFRT showed the highest association with diagnosis among other compositions (effect size = 0.466).

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Table 2. Results of multivariate GLM analysis with all biomarkers as dependent variables and thalassemia diagnosis versus controls as the explanatory variable while adjusting for sex and age.

Tests	Dependent variables	Explanatory variables	F	df	*p*-value	Partial η^2
Multivariate	All Biomarkers	Diagnosis	17.11	6/116	<0.001	0.533
		Age	0.17	6/117	0.985	0.011
		Sex	1.24	6/118	0.147	0.091
		#Transfusion	0.31	6/119	0.931	0.02
		Duration of disease	0.58	6/120	0.743	0.037
Between-Subjects Effects	Iron	Diagnosis	115.54	1	<0.001	0.541
	TIBC	Diagnosis	26.29	1	<0.001	0.212
	UIBC	Diagnosis	41.19	1	<0.001	0.296
	TS%	Diagnosis	109.39	1	<0.001	0.527
	Transferrin	Diagnosis	26.29	1	<0.001	0.212
	FRT	Diagnosis	101.81	1	<0.001	0.510
	HEPC	Diagnosis	13.84	1	0.005	0.138
	ERFE	Diagnosis	19.13	1	<0.001	0.163
	zERFE-zHEPC	Diagnosis	12.96	1	0.008	0.129
	zERFE-zHEPC+zFRT	Diagnosis	42.09	1	<0.001	0.300
	zERFE+zFRT	Diagnosis	85.41	1	<0.001	0.466

Table 3 shows the intercorrelation matrix among different biomarkers. As expected, the ISPs were correlated with each other (p<0.001) since all calculate from the serum iron concentration and TIBC values. Serum FRT is significantly correlated with serum iron and UIBC (p<0.05).

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ERFE is inversely correlated with HEPC level (p<0.05), while no direct correlation was detected between the ERFE and HEPC with the other measured ISPs. The number of transfusion and duration of the disease are correlated with age.

Table 3. Intercorrelation matrix among biomarkers.

Biomarkers	Age	#Transfusion	Duration of disease	Iron	TIBC	UIBC	TS%	Tf	FRT	HEPC
#Transfusion	0.43**									
Duration of disease	0.71**	0.65**								
Iron	-0.14	0.29*	-0.13							
TIBC	-0.08	-0.12	-0.05	0.48**						
UIBC	0.01	-0.02	0.03	-0.65**	0.27*					
TS%	-0.04	0.17	-0.04	0.81**	-0.04	-0.97**				
Transferrin	-0.08	-0.07	-0.05	0.48**	0.99 **	0.27*	-0.04			
FRT	-0.01	0.11	0.11	0.19	-0.076	-0.25*	0.21	-0.08		
HEPC	0.09	-0.14	0.04	-0.16	-0.119	0.01	-0.02	-0.12	0.16	
ERFE	0.07	-0.02	0.11	0.18	0.14	-0.15	0.15	0.14	0.15	-0.27*

* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

The binary logistic analysis results of the biomarkers are presented in Table 4. We considered β-TM as the dependent variable, healthy controls as the reference group, and biomarkers as explanatory variables. Only the parameters that showed a significant power prediction (p<0.05) are presented in the table. Binary logistic regression predicts the odds of being a patient with β-TM based on the values of the independent biomarkers (predictors). The odds are defined as the ratio of the (probability of being a patient with β-TM)/ (probability of being a healthy individual). According to the results, ERFE, HEPC, FRT, and transferrin biomarkers can be used to predict β-TM significantly. The powers of prediction from the regressions are close with each other and follows the order:

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ERFE (OR=1.033) > HEPC (OR=1.012) > transferrin (OR=1.010) >FER (OR=1.002).

Table 4. Binary logistic regression analysis to determine predictors of β-TM.

Dependent Variables	Explanatory variables*	B (SE)	Wald	df	*p*-value	OR	95% CI for OR
β-TM vs. HC	ERFE	0.032(0.006)	25.32	1	<0.001	1.033	1.02-1.046
β-TM vs. HC	HEPC	0.012(0.004)	11.669	1	0.001	1.012	1.005-1.019
β-TM vs. HC	FRT	0.002(0.001)	30.127	1	<0.001	1.002	1.001-1.003
β-TM vs. HC	Transferrin	0.010(0.002)	34.041	1	<0.001	1.01	1.007-1.013

Abbreviations: OR, odds ratio; CI, confidence interval; SE, standard error

* Confounding variables (age and sex) were added. The regression was adjusted .

The results of ROC analysis including area under the curve (AUC), cut-off scores, and Youden's J statistics for the ERFE and its z-unit weighted composite scores with other predictor biomarkers are presented in Table 5, and its graphical representation is shown in Fig. 1.

Table 5: ROC-AUC analysis for ERFE and its composition for diagnosis of β-TM.

Biomarker	AUC (SE)	Youden's J statistic	Sensitivity (%)	Specificity (%)	p-value
zERFE-zHEPC	0.821 (0.043)	0.58	74	80	<0.001
ERFE	0.924 (0.025)	0.81	80	100	<0.001
zERFE+zFRT-zHEPC	0.942 (0.022)	0.86	85	95	<0.001
zERFE+zFRT	1.000 (0)	1.00	100	100	<0.001

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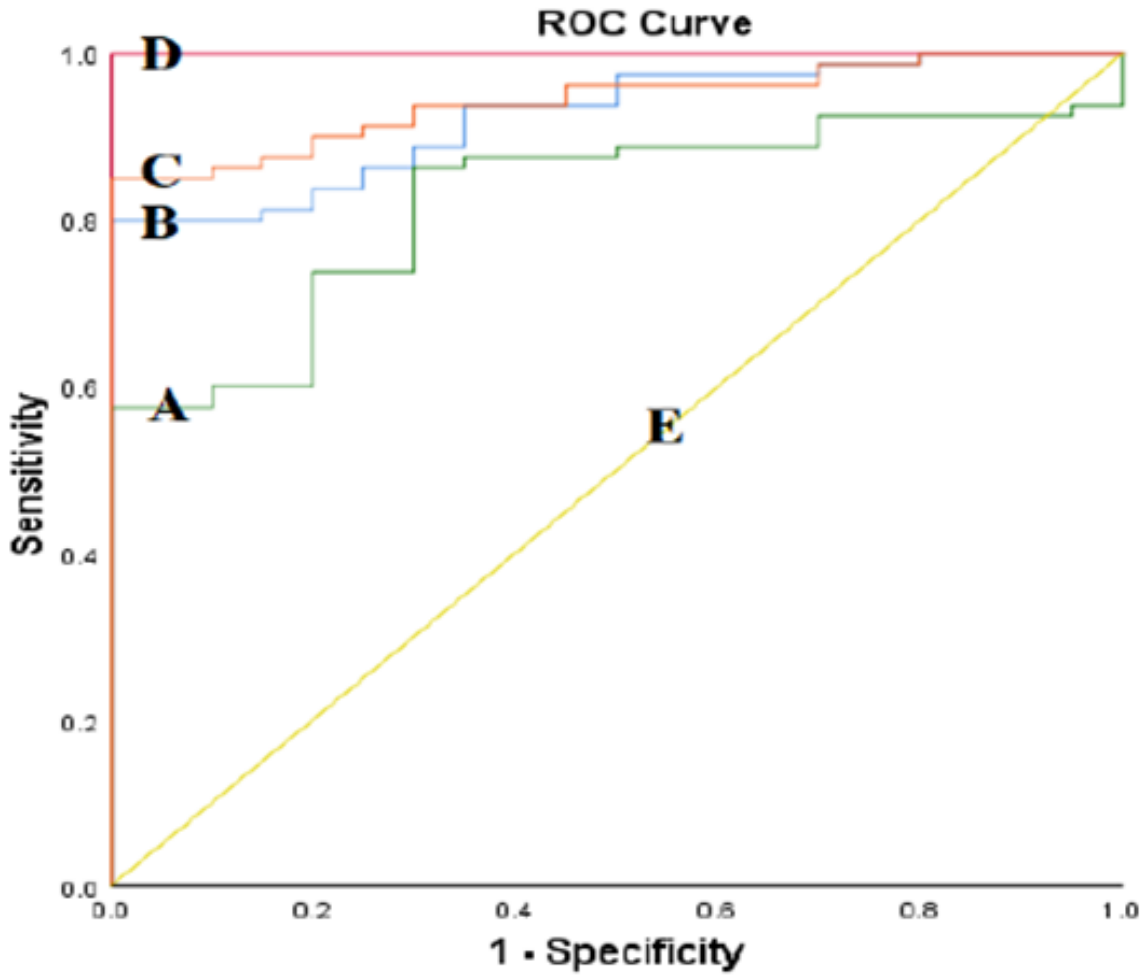

Figure 1. ROC analysis curve for A) zERFE-zHEPC, B) ERFE, C) zERFE+zEREF-zHEPC, and, D) zEREF+zFRT parameters as predictors for β-TM in compared with E) reference line.

Discussion

In the present study, we measured levels of ISPs and two main iron regulatory hormones in the serums of β-TM patients and healthy controls. Significant increase (p<0.05) in all iron indices were observed in patients with thalassemia compared with the control group, except for UIBC. According to the calculation formula (UIBC = TIBC – serum iron concentration), the lower observed UIBC is mainly due to the approximately 2.5 fold increase in the serum iron level that occurred in patients. Since all subjects were free from any obvious inflammation or infection (CRP < 6 mg/ml), these results indicated the apparent state of iron overload in β-TM patients.

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Furthermore, The intercorrelation matrix among different biomarkers indicated the expected correlation of ISPs (that all are derived from iron and TIBC) with each other. Among all the analysed indices, FRT displays the highest increase in patients' blood (~ 11 fold). FRTs are among the major iron-containing proteins, and its serum level is directly proportional to total body iron stores. This protein functions as one of the endogenous antioxidants that prevents the harmful effects of iron-catalysed oxidative stress by reducing the influx of iron into the plasma [22]. In the iron overload condition, like β-TM, hemochromatosis or inflammation, the majority of the excess iron sequesters in FRT [23]. Very high FRT levels that are often measured in β-TM suggest this analysis as a monitoring tool for iron load in this patients, especially when other modalities to assess iron overload are not readily available [24, 25]. Moreover, serum FRT that is considered as the leading indicator of iron overload has a significant negative correlation with UIBC. Serum FRT level has been found as predictors of cardiac risk in patients with β-TM and lowers FRT concentration reported to be favourable for longer survivals [26]. Furthermore, its regular measurement was suggested to be useful in monitoring the risk of chelation therapy toxicity, especially in children [27].

As it was expected, plasma levels of the two main regulators of iron homeostasis, HEPC and ERFE hormones, were changed significantly in β-TM. The inverse correlation of ERFE with HEPC in β-TM patients is shown in table 3. Increased but inefficient erythropoiesis in thalassemia mediates the up-regulation of ERFE and down-regulation of HEPC (in this study, 3.1 and 2.2 folds of increase and decrease, respectively).

Kautz and colleagues identified the ERFE as an erythroid factor that mediates HEPC suppression in *Hbb*$^{th3/+}$ mice with thalassemia intermedia and contributes to iron overload [28]. However, the molecular mechanism underlying the suppression of HEPC by ERFE was clarified

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much more recently by Arezus and colleagues. They found that ERFE suppresses HEPC via inhibition of hepatic BMP/SMAD signalling by impairing the evolutionary closely related BMP subgroup of BMP5, BMP6, and BMP7 [29]. Some studies suggested HEPC therapy and ERFE suppression to the prevention and treatment of iron overload in disorders including β-TM [30-32].

All studied biomarkers highly associated with the diagnosis; however, the iron level has the strongest association which followed by TS%, FRT, and zERFE+zFRT composition.

Binary logistic regression analysis indicated that all tested explanatory variables (ERFE, HEPC, transferrin, and FER) significantly predict β-TM, while ERFE has a little more power for prediction (OR=1.033). The ROC analysis was employed to determine the sensitivity and specificity of the different composition of ERFE for diagnosis of β-TM, while AUC was calculated to determine the diagnostic accuracy of each composition. We computed three relevant z unit-weighted composite scores; zERFE-zHEPC, zERFE+zFRT-zHEPC, and zERFE+zFRT for analysis and he zERFE+zFRT composition indicated a full diagnostic ability. ROC analysis is used in clinical epidemiology to quantify how accurate medical diagnostic tests (or systems) can discriminate between two states, typically referred to as patients and control [33]. The obtained result presented a new useful diagnostic tool for β-TM with a specificity of 100%.

In conclusion, our finding indicated the correlation between different ISPs, FRT (as the leading predictor of iron overload) and tow main iron regulatory hormones. The results were extracted from Iraqi patients, and maybe influenced by the used therapeutic intervention, but it is suggested that it could be pretty generalized. These outcomes may also be helpful for further studies in other iron overload complications.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

We acknowledge the staff of Al-Zahraa Teaching Hospital for their help in the collection of samples. We also acknowledge the work of the high-skilled staff of Asia Laboratory in the estimation of biomarkers level.

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