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Original article

The modulation of plasma levels of dopamine, serotonin, and brain-derived neurotrophic factor in response to variation in iron availability

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Abstract. *Background and aim:* In the context of iron deficiency anemia, central dopamine, serotonin, and brain-derived neurotrophic factor (BDNF) are intensively investigated. However, peripheral isoforms are poorly investigated. This study aimed to investigate the modulation of plasma levels of dopamine, serotonin, and BDNF among children with iron deficiency anemia. *Methods:* A total of seventy-three iron-deficient (n=38) and iron-sufficient (n=35) children were included in the study. Twenty-nine subjects were showing clinical presentations and were diagnosed with iron deficiency anemia and forty-four were asymptomatic normal children. Plasma levels of dopamine, serotonin, and brain-derived neurotrophic factor were determined by enzyme-linked immunosorbent assay. *Results:* As compared to corresponding levels among control subjects, Anemic subjects were having significantly higher plasma dopamine and lower plasma brain-derived neurotrophic factor levels. A significant linear and monotonic association of plasma dopamine and brain-derived neurotrophic factor with hemoglobin concentration ($r=-0.520$, $P < 0.001$ and $\rho = 0.411$, $P = 0.001$), respectively. Furthermore, there were significantly higher plasma serotonin levels among iron-deficient subjects with a significant inverse linear association with serum ferritin levels ($r = -0.337$, $P = 0.005$). *Conclusions:* Iron deficiency anemia is associated with the modulation of peripheral dopamine, serotonin, and brain-derived neurotrophic factor. Upregulation of dopamine and downregulation of brain-derived neurotrophic factor are correlated to the anemic status. The upregulation of plasma serotonin levels is iron-dependent and, probably, is attributed to the impairment of its metabolic fate. Further investigation is required to explore the pathophysiological and clinical association of these peripheral biomolecules in the context of iron deficiency anemia. (www.actabiomedica.it)

Key words: Iron Deficiency, Anemia, Dopamine, Serotonin, Brain-derived neurotrophic factor

Introduction

Iron is a micro-nutritional mineral that is required for a wide variety of vital metabolic activities and biochemical reactions (1). Most iron contents are incorporated in the structure of hemoproteins; hemoglobin and myoglobin (1, 2). Hemoglobin is the functional unit in erythrocytes and is a determinant of oxygen-carrying capacity (3). Due to the high erythrocytic turnover rate and their iron abundance; iron deficiency (ID) results in several systemic health impacts of which anemia (IDA) is the earliest and the most common (4). Children are among the age groups with high iron demand, and so are one of the most vulnerable to ID clinical complications (1). According to the world health organization (WHO), approximately, 30% to 50% of anemia during childhood is IDA (5). Hence, there are various highlighted impacts of ID and IDA on a child's life.

Hypoxia and diminished iron availability are major determinants of the clinical manifestations of IDA (6). Iron deficiency is associated with an increased risk of developing serious clinical impacts (7). ID and IDA were evident to be associated with cognitive deficit and physical incompetence that were suggested to be attributed to either hypoxia or iron unavailability (6). Central neurotransmitters are key intermediates in the development of cognitive outcomes, emotional behaviors, and motor coordination in response to iron deficiency. Among these neurotransmitters; monoamine (dopamine DA and serotonin 5-HT) and neurotrophins (brain-derived neurotrophic factor (BDNF) were investigated (8).

Peripheral dopamine, serotonin, and BDNF function as paracrine hormones with systemic regulatory roles in body homeostasis (9-12). In the peripheral, dopamine is integrated into the function of the immune system and the hemodynamic balance while serotonin is integrated into the function of the digestive tract and platelet coagulation (9-12). The modulation of plasma levels of these biomolecules in the context of iron ID and IDA is poorly investigated. Here in this study, we aimed to investigate the plasma levels of these hormones in accordance with iron deficiency and corresponding anemia. This may define the contribution of these hormones to the clinical features and complications of the disease. Considering the significant variation in iron hemostasis, diagnostic features, and neuronal heterogeneity with age; this study was focused on school-aged children.

Patients and methods

Patients and sample collection

Seventy-three subjects (N=73) with an age range of 5 to 12 years old were included in the study. Subjects were randomly selected from children who were visiting a pediatrics clinic at a governmental children's hospital and were diagnosed with iron deficiency Anemia in accordance with the diagnostic criteria as defined by the world health organization (WHO). Thirty-eight (n=38) children were found to be iron-deficient and thirty-five were iron-sufficient (n=35). Based on the WHO diagnostic criteria; twenty-nine subjects were diagnosed with iron deficiency anemia and forty-four were asymptomatic or normal children. Children with other hematological disorders (such as thalassemia and anemia of chronic disease), chronic inflammatory disease, or current infection were excluded. Furthermore, children who had been taking iron supplements within the last three months before enrollment day were excluded from the study.

Parents/Guardians of the study subjects were informed about the study and upon their approval, their children were included in the study after signing a consent form in that regard. This study was reviewed and approved by an institutional review board (IRB approval Number:12-119-2018).

Three blood samples were withdrawn from each participant by venipuncture: EDTA blood for hematological complete blood count (CBC), plain tube for serum ferritin measurement, and sodium heparinized blood for the measurement of plasma levels of dopamine, serotonin, and BDNF. Once separated by centrifugation at 4500 rpm for eight minutes, serum and plasma samples were stored frozen at -80°C until analyzed.

Complete blood count and serum ferritin level

CBC analysis was conducted to confirm anemic status among study subjects and to categorize them, accordingly, into anemic and non-anemic subjects. Red cell indices included red cells count, hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC), and red cells distribution width (RDW). CBC analysis was conducted on EDTA blood samples using an automated cell counter instrument (Beckman Coulter LH 780 Analyzer).

Serum ferritin levels for study subjects were also evaluated to confirm the diagnosis of IDA and to categorize study subjects into iron deficient and iron non-deficient subjects. Serum samples were obtained from plain blood tubes by centrifugation at 4500 rpm for 8 minutes and processed for serum ferritin levels using a commercially available automated instrument (Beckman coulter Access2, Europe).

Plasma levels of dopamine, serotonin, and BDNF

Plasma samples from heparinized blood were separated and used to determine the plasma levels of the study markers. Enzyme-linked immunosorbent assay (ELISA) was conducted using commercially available kits for the measurement of plasma dopamine (MyBioSource, USA), serotonin (Abcam, UK), and BDNF (Abcam, UK). Technical procedures were according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using the statistical package for the social sciences (SPSS). Independent Student's t-test was used for comparative purposes and the results were considered significant when the P value was less than (0.05). A correlation analysis was conducted using parametric Pearson's (r) and nonparametric Spearman's (Rho) correlation analysis. Graphs were prepared using GraphPad Prism 6 software.

Results

Study subjects were randomly selected children with an age range of 5 to 12 years old (average 8.85 ± 0.23 years). Among whom, thirty-seven (37) subjects were males and 36 were females (the ratio is 1:1). Physical examination of subjects has revealed that 60% (n=45) of them were having no clinical presentation of anemia, and 40% (n=28) were showing evident signs and symptoms including skin and conjunctiva paleness, shortness of breath, and loss of appetite. Advanced clinical features, including koilonychia and angular cheilitis, were presented in only three patients (n=3, 4.3%).

Per the WHO recommendations, anemia is diagnosed by having a Hb lower than a cut-off value that varies by age and gender (13). Adult males and females are diagnosed as being anemic when having a Hb lower than 12g/dL and 13 g/dL, respectively (14). During childhood, the cut-off value is postulated to be 11.5 g/dL and 12.0 g/dL for children aged 5-11 and 12-15 years, respectively (13). It is worthy to mention that the diagnostic cut-off value of Hb may vary with ethnicity, geographic ancestry, and body demands (15). Therefore, to validate the cut-off Hb among study subjects, the descriptive analysis revealed that the average Hb among clinically symptomatic anemic was 11.4 ± 0.3 g/dL in comparison to a corresponding average concentration of 13.0 ± 0.1 g/dL among control subjects ($p<0.001$). Based on that, we have concluded that a Hb of 12.0 g/dL is a reliable cut-off value among our study subjects to classify them as anemic patients versus non-anemic subjects.

As compared to serum iron, transferrin saturation, and total iron-binding capacity, serum ferritin is a reliable diagnostic hallmark of iron deficiency as it is evident to be clinically correlated to iron stores in the bone marrow (16). During childhood, the normal range of serum ferritin is 10-55 ng/mL (17). The typical cutoff value of 12-20 ng/mL is proposed to be a diagnostic of ID and IDA (18). However, it has been reported that normal serum ferritin may coexist with depleted iron stores in the bone marrow (19). This may deceive the diagnosis of iron deficiency anemia that may remain unrecognized for a long time. Herein, as shown in Table 1, we have reported that anemic subjects were having an average serum ferritin concentration of 16.5 ± 1.7 ng/mL as compared to 25.2 ± 2.5 ng/mL ($P < 0.05$). Based on these findings,

Table 1. Demographic features, red cell indices of CBC analysis, and serum ferritin levels among study subjects.

Parameter	Non-anemic (n=38)	Anemic (n=32)	P value
Age (years)	9.0 ± 0.4	8.6 ± 0.3	0.41
Hb † (g/dL)	13.3 ± 0.1	11.2 ± 0.3	<0.001
RBC †† (×106/ml)	5.15 ± 0.10	4.90 ± 0.11	0.04
Hct ‡ (%)	39.8 ± 0.5	34.4 ± 0.7	<0.001
MCV § (fL)	77.6 ± 0.9	70.8 ± 1.3	<0.001
MCH §§ (pg)	25.9 ± 0.3	23.2 ± 0.6	<0.001
MCHC ¶ (g/dL)	33.4 ± 0.3	32.8 ± 0.4	0.24
RDW # (%)	13.4 ± 0.1	14.9 ± 0.3	<0.001
Ferritin (ng/mL)	25.2 ± 2.5	16.5 ± 1.7	0.03

Comparative analysis of Hb, RBC count, Hct, MCV, MCH, RDW and serum ferritin level. between anemic and non-anemic subjects. †Hb: Hemoglobin concentration, ††RBCs: red cell count, ‡Hct: Hematocrit, §MCV: Mean corpuscular volume, §§MCH: Mean corpuscular hemoglobin, ¶MCHC: Mean corpuscular hemoglobin concentration and #RDW: red cells distribution width. Results are represented as mean ± SEM.

we have concluded that the cutoff value of 20 ng/mL is valid for our study subjects. The significant difference in average Hb among iron-deficient (11.8 ± 0.3 g/dL) and non-iron deficient subjects (12.9 ± 0.2 g/dL) (P < 0.001) may support the validity of the cutoff values of Hb and serum ferritin concentration that we concluded in our study.

As shown in Table 1, the demographic criteria as well as the results of CBC and serum ferritin levels among study subjects are illustrated. In addition to the previous descriptive analysis, a strong significant association was evident between Hb and Hct (r = 0.863, P < 0.001), RDW (r = - 0.798, P < 0.001), MCH (r = 0.643, P < 0.001) and MCV (r = 0.682, P <0.001). Moderate associations were observed with RBC's count and MCHC (r = 0.432, P < 0.001) and (r = 0.352, P = 0.003), respectively.

To validate the diagnosis of IDA, a correlation analysis was conducted to evaluate the association of serum ferritin with the clinical severity and red cell indices. As illustrated in Table (2), spearman's correlation analysis has revealed a significantly strong correlation between serum ferritin and the clinical severity of anemia. Furthermore, a significantly strong direct correlation was found between serum ferritin and most red cell indices including Hb, Hct, MCV, MCH, and RBCs count except for RDW which was inversely correlated. No significant correlation was detected between MCHC and neither the clinical severity nor the serum ferritin levels.

A comparative analysis of plasma dopamine, serotonin, and BDNF levels among study subjects was conducted. As shown in Figure 1, the average dopamine level among anemic patients was 904.9 ± 102.0 pg/mL, which is significantly higher than the corresponding average levels among control subjects (668.8 ± 41.7 pg/mL, (P = 0.02)). Furthermore, a significant difference was shown in the average levels of BDNF with 110.8 ± 19.9 pg/mL and 51.5 ± 10.4 pg/mL among control non-anemic subjects and anemic patients, respectively (P = 0.02).

To analyze the association between ID and study parameters, study subjects were categorized based on their serum ferritin level; known as a determinant of the extent of ID (20). Accordingly, 50% of the subjects (n=35) were defined as iron deficient with serum ferritin levels of up to 20μg/L with an average serum level of 11.1 ± 0.9 μg/L. The remaining 50% of subjects (n=35) were defined as having normal levels of serum ferritin with an average of 32.7 ± 2.4 μg/L. The difference in average serum ferritin levels between the two groups is statistically significant (P < 0.001).

As shown in Figure 2, a comparative analysis of the study subjects has revealed that the average plasma serotonin level among the iron-deficient group was 784.9 ± 71.0, which is significantly higher than its corresponding average level among iron sufficient subjects with an average concentration of 691.8 ± 48.1 (P < 0.001). In regard of dopamine and BDNF, there were no significant differences in their average plasma levels between the two groups (P > 0.05).

To investigate the association of anemia and iron deficiency to plasma levels of study parameters, Pearson's correlation analysis was conducted. As illustrated in Table 3, there was a significant inverse linear relationship between plasma dopamine levels and Hb concentration. On the other hand, a significant inverse relationship was evident between plasma serotonin levels and serum ferritin. Furthermore, partial

Table 2. Correlation analysis of clinical symptoms and serum ferritin levels with red cell indices.

Parameter	Spearman's Corr.	Ferritin	Hb†	RBCs††	Hct‡	MCV§	MCH§§	MCHC¶	RDW
Clinical status	*r*	-0.580	-0.513	-0.271	-0.617	-0.482	-0.392	-0.059	0.474
	P value	<0.001	<0.001	0.023	<0.001	<0.001	0.001	0.627	<0.001
Ferritin	*r*	---	0.469	0.256	0.536	0.411	0.268	-0.079	-0.401
	P-value		<0.001	0.032	<0.001	<0.001	0.025	0.517	0.001

Spearman's (*rho*) correlation coefficient (r) for the relationship of red cell indices with the clinical status and serum ferritin levels among study subjects. †Hb: Hemoglobin concentration, ††RBCs: red cell count, ‡Hct: Hematocrit, §MCV: Mean corpuscular volume, §§MCH: Mean corpuscular hemoglobin, ¶MCHC: Mean corpuscular hemoglobin concentration and #RDW: red cells distribution width.

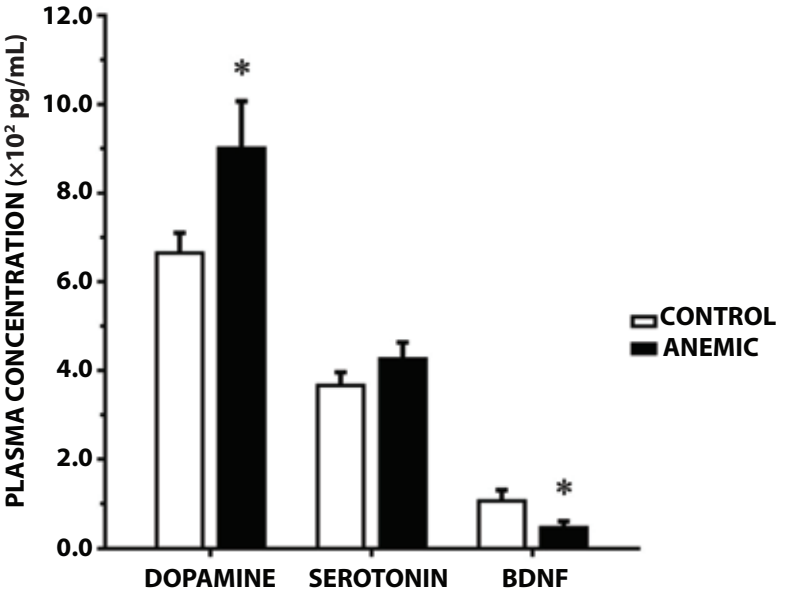

Figure 1. A comparison of average plasma levels of dopamine, serotonin, and BDNF between anemic subjects and non-Anemic subjects. Results are represented as mean ± SEM, *indicates a significant comparative analysis between anemic and control subjects.

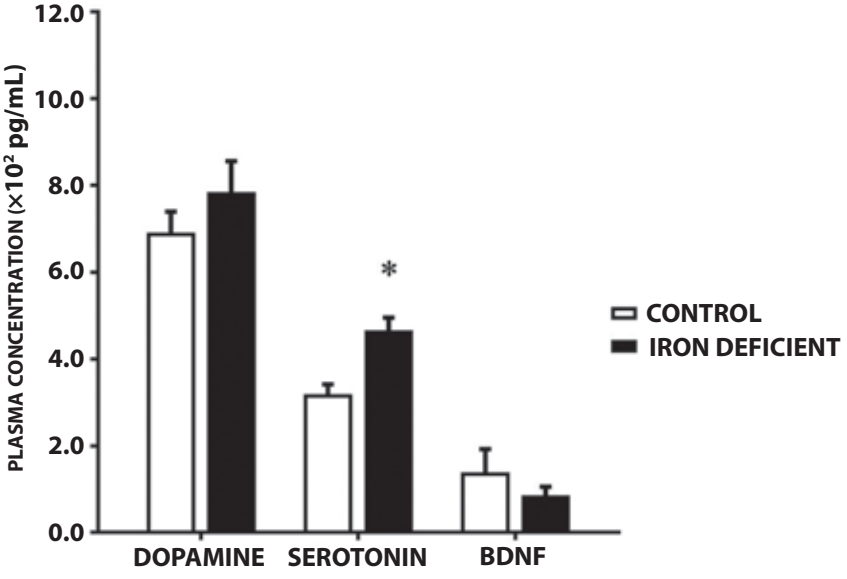

Figure 2. A comparison of average plasma levels of dopamine, serotonin, and BDNF between iron-deficient control subjects. Results are represented as mean ± SEM, *indicates a significant comparative analysis between iron-deficient and control subjects.

correlation analysis has revealed the significant association between Hb concentration and plasma dopamine levels persisted even when controlling for serum ferritin ($r = -0.587$, $P < 0.001$). Similarly, the significant inverse association was evident even when controlling for Hb concentration ($r= -0.362$, $P = 0.003$). Based on the absence of the linear association between plasma BDNF and hemoglobin concentration; nonparametric

Table 3. Correlation analysis of plasma levels of dopamine, serotonin, and BDNF with elected hematological red cell indices and serum ferritin levels among study subjects.

Parameters	Correlation	Hb †	Hct ‡	RDW §	Ferritin
Dopamine	*Pearson's (r)*	-0.520	-0.378	0.532	-0.221
	P-value	< 0.001	0.004	< 0.001	0.10
Serotonin	*Pearson's (r)*	-0.018	-0.144	0.024	-0.337
	P-value	0.88	0.243	0.85	0.005
BDNF ¶	*Pearson's (r)*	0.222	0.206	-0.172	0.230
	P-value	0.08	0.10	0.17	0.07

Parametric Pearson's (r) correlation analysis for the association of Hb, Hct, RDW and serum ferritin with plasma levels of dopamine, serotonin and BDNF. †Hb: Hemoglobin concentration, ‡Hct: Hematocrit, and §RDW: red cells distribution width. ¶BDNF: Brain-derived neurotrophic factor.

Spearman's correlation analysis has revealed a direct monotonic association with a correlation coefficient of $\rho = 0.411$, $P = 0.001$.

To further analyze the association between serum dopamine and anemia as well as serum serotonin levels and iron deficiency; linear regression analysis was conducted to define the predictive values of Hb and serum ferritin levels of plasma dopamine and serotonin, respectively. Results have revealed the satisfaction of our data sets in terms of the required assumptions for regression analysis and subsequently the reliability and validity of analysis outputs. In regard to the prediction of dopamine level; Hb is having a statistically significant strong predictive value for plasma dopamine level (a standardized beta coefficient = - 0. 517, P = 0.000). On the other hand, serum ferritin level is moderately predictive of plasma serotonin level with a standardized beta coefficient = -0.397, P = 0.003). Considering the sample size of our study population, were investigated the statistical and clinical reliability, of our comparative and correlative analysis, using the analysis of effect size and observed power. Accordingly, the effect size and observed power analysis revealed a partial Eta squared value of 0.86 and a power value of 0.91, respectively. The obtained partial Eta squared, and power values imply a clinical reliability of our data and a statistical validity of our analysis.

Discussion

Hormonal modulation in the context of iron deficiency anemia was evident to be implicated in the clinical presentation of the disease (21). In addition to being neurotransmitters with several neurological functions, Dopamine, serotonin, and BDNF are paracrine hormones with a wide variety of systemic impacts (9, 11, 12). This study aimed to investigate the modulation in plasma levels of dopamine, serotonin, and BDNF as a consequence of IDA. To achieve our goals, study subjects were categorized based on their hematological status as anemic and non-anemic. Red cell indices were consistent with the defined criteria of IDA. However, as being the least sensitive red cell index to IDA in children (22); MCHC was an exception with no significant difference between study groups

The diagnosis of IDA is occasionally mysterious where the development of the clinical presentation and diagnostic features may remain unrecognizable up until the advanced stage of iron deficiency (4). It is worthy to mention that, in certain cases, iron deficiency may not be associated with the development of anemia (19). Still, regardless of the type of anemia, reduced levels of Hb and Hct are well-established designations for anemia (23). Furthermore, a reduced MCV is a definition of microcytosis which is a characteristic feature of erythrocytes from patients with iron deficiency anemia and is used as a surrogate marker for an early diagnosis of the disease (24). The normal value of mean corpuscular volume (MCV) varies with age with an average of 86 fL for children aged 6 to 12 years old and microcytosis is defined by having an MCV value below the cutoff of 77 fL (25).

In turn, RDW is a sensitive and reliable parameter that aids in the differential diagnosis of microcytic hypochromic anemia including IDA (26). Among all other red cell indices, we have reported the strongest

correlation of Hb, Hct, and RDW to the clinical presentation and serum ferritin. Accordingly, for comparative purposes and correlation analysis, we used these red cell indices as diagnostic markers that define the extent and severity of the disease.

Following to the categorization of study subjects based on anemia development and iron deficiency status, plasma levels of dopamine, serotonin, and BDNF were determined. The reported higher level of plasma dopamine among anemic subjects as well as its significant correlation with red cell indices may define the upregulation of peripheral dopamine as a possible consequence of the reduction in red cell mass and consequent hypoxia. In response to hypoxia, a direct positive association was reported between hemoglobin concentration and modulation of vascular tone to counteract impaired perfusion (27). Peripheral dopamine was identified as a key modulator of vascular tone in health and disease (28).

On the other hand, the significant reduction of plasma BDNF, as well as its monotonic association with the red cell indices and the clinical presentation of anemia, may define the downregulation of peripheral BDNF as an intermediate consequence of IDA that may, significantly, contributes to the clinical outcomes. A considerable portion of peripheral BDNF is originated from the CNS, as it can bi-directionally cross the blood-brain barrier (29). Unlike dopamine and serotonin, the peripheral BDNF level is relevant to its corresponding level in the CNS (30). Functionally, BDNF contributes to mood control and, therefore, its reduced level is implicated in depression disorders (31, 32). Depression is a clinical consequence of IDA and is correlated to the severity of the disease (32). Moreover, earlier studies on animal rat models, have defined that neonatal IDA is directly implicated in the decrease of BDNF level and activity in the hippocampus of the brain resulting in impaired neuronal differentiation and long-term cognitive dysfunction (33).

Unlike dopamine and BDNF, it is obvious that anemia had no significant impact on plasma serotonin levels. However, the influenced plasma serotonin levels among iron-deficient subjects, as well as its significant association with serum ferritin level, are suggestive of the impact of iron deficiency on peripheral serotonin. The majority of body serotonin is in the peripheral pool and is produced by enterochromaffin cells in the gut (34, 35). Several studies have reported elevated plasma serotonin levels in the context of a variety of pathological situations. Authors have proposed several mechanisms, of which, the downregulation of serotonin transporter (SERT) density was the most probable (36). Tryptophan, an amino acid substrate for serotonin synthesis, availability is a determinant of serotonin hemostasis in the gut and the central nervous system (37). In a study by J Winninger (2019), a directly proportional correlation of peripheral tryptophan, and its degradation product, with serum ferritin was reported (38). In response to ID, authors have suggested an accelerated cellular uptake and metabolic conversion of tryptophan, into serotonin. Thus, their findings may agree with ours. It is worthy to mention here that tryptophan metabolism into serotonin is mediated by an iron-dependent tryptophan hydroxylase (38). However, iron unavailability impairs tryptophan hydroxylase activity when iron level goes beyond 2×10^{-12} M (39). This is an extremely iron deficiency that none of our study subjects achieved.

Functionally, circulatory serotonin is implicated in a variety of physiological activities including the induction of platelet aggregation, modulation of vascular tone, and gastrointestinal motility (40). These effects are, primarily, mediated by serotonin receptors (SERT) expressed on the platelet surface and vascular smooth muscles, respectively (40). Iron deficiency anemia has been evident to be associated with clinical presentations including impaired platelet aggregation, pulmonary hypertension, and chronic inflammatory disease symptoms (40, 41). Accordingly, the upregulation of serotonin, in the context of iron deficiency anemia, may contribute to the development of these clinical presentations of the disease.

In conclusion, it is evident here that ID and IDA are associated with a modulation of the peripheral plasma levels of dopamine, serotonin, and BDNF. Dopamine upregulation and BDNF downregulation are correlated with the anemic status. On the other hand, serotonin upregulation is iron-dependent and is probably a result of peripheral serotonin's impaired metabolic fate. Further investigation is required to explore the pathological mechanisms and the clinical impacts of included peripheral biomolecules in iron deficiency

anemia. We understand that sample size is a major limitation in our study. Therefore, a larger sample size, with the inclusion of other age groups, is required to validate our obtained findings and confirm the modulation of investigated biomolecules in the context of iron deficiency and its associated anemia.

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Authors Contributions: M. B. A. and M. M. O. conceived the study conception and design. M. H. A., M. M. O. and M. B had contributed into recruitment of participants, clinical assessment, and sample collections. M. H. A. conducted experimental work and laboratory analysis. All authors had significant contribution to data analysis and results interpretation. M. B. A. conducted the statistical analysis and wrote the manuscript. All authors had reviewed, edited, and approved the final draft of the manuscript.

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