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Impact of Oral Iron Therapy on Oxidative Stress and DNA Damage in Women of Reproductive Age

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ABSTRACT

Objective: Iron deficiency anemia (IDA) is a condition that increases morbidity and mortality. This study investigated changes in oxidative stress, DNA damage, and hepcidin levels following therapy in individuals with IDA.

Materials and Methods: The patient group consisted of 39 females diagnosed with IDA, and 36 age- and sex-matched controls were incorporated into our study (18-49 years). The patient group was treated with daily oral ferrous fumarate (304.2 mg, approximately 100 mg elemental iron) for one month. Measurements of total oxidant status (TOS), total antioxidant status (TAS), oxidative stress index (OSI), DNA damage (using the Comet assay), and hepcidin levels were conducted before and during therapy and compared between the patient and control groups.

Results: Oxidative stress indicators and DNA damage assessed with the Comet assay were elevated in individuals with IDA ($p=0.018$) and decreased following treatment ($p=0.002$). TAS was reduced in individuals with IDA ($p=0.02$) but increased after treatment ($p<0.001$). Although TOS levels were elevated in individuals with IDA, the difference was not statistically significant ($p=0.10$); however, TOS levels decreased after treatment ($p=0.02$). Hepcidin levels were significantly lower in individuals with IDA ($p<0.001$) but increased during therapy ($p<0.001$).

Conclusion: This study demonstrates that just one month of oral iron therapy can reversibly improve oxidative stress and DNA damage. Hepcidin plays a critical role in maintaining iron homeostasis.

Keywords: DNA damage, hepcidin, iron deficiency anemia, oral therapy, oxidative stress.

INTRODUCTION

Anemia is a significant global health issue, affecting 24.8% of the world's population.[1] About 50% of anemia cases are attributed to iron deficiency.[2] Iron deficiency leads to the synthesis of reactive oxygen species (ROS), disrupting the balance between antioxidants and promoting

oxidative stress. Consequently, oxidative stress results in structural impairments in proteins, lipids, and DNA.[3] Additionally, iron deficiency impairs mitochondrial electron transport chain (ETC) activity, leading to ROS production and triggering a cellular energy crisis.[4]

Hepcidin plays a crucial role in iron homeostasis. In iron deficiency anemia (IDA), hepcidin levels are diminished. This reduction promotes dietary iron absorption and prevents iron overload.[5]

DNA damage is assessed by analyzing the effects of genotoxic, cytotoxic, and oxidative stress.[6] DNA damage and repair in eukaryotic cells can be assessed using the Comet test, also known as single-cell gel electrophoresis.[7] Furthermore, assessing total antioxidant status (TAS) and total oxidant status (TOS) provides valuable insights into oxidative stress in IDA. Iron supplementation is the most important strategy for preventing IDA.[8] Research has shown that iron treatment can enhance antioxidant defenses. However, it may also increase the formation of free radicals, leading to oxidative stress.[9]

Most research has focused on investigating cell cultures in IDA. Additionally, previous studies on IDA predominantly target the pediatric population. This study, therefore, aims to assess DNA damage and oxidative stress throughout the body using TAS, TOS, oxidative stress index (OSI), and the Comet assay in reproductive-age women with IDA. Furthermore, we aim to highlight the role of hepcidin in maintaining iron homeostasis during oral therapy.

MATERIALS AND METHODS

This research was funded by Pamukkale University (Project Number: 2020TIPF028) and approved by the Ethics Review Board of Pamukkale University (Date: 03.11.2020; Decision no: 60116787-020/66544). The study was conducted in accordance with the Helsinki Declaration, and informed consent was obtained from each participant.

Reference values for the diagnosis of IDA are hemoglobin (Hb) <12 g/dL and ferritin <15 mg/L.[10] We included 39 female patients with IDA and 36 age- and sex-matched controls in the study, conducted at Pamukkale University Faculty of Medicine Hematology Policlinic. The patient group received 304.2 mg of oral ferrous fumarate (approximately 100 mg elemental iron) daily, and measurements were taken after one month of therapy. Participants in the patient group did not have diabetes, hypertension, renal failure, cerebrovascular disease, gastrointestinal tract illness, coronary artery disease, chronic liver disease, hematologic or solid organ malignancies. Members of the control group were healthy individuals without known diseases or medication usage. A kit was used to measure TOS, TAS, and hepcidin levels.

KEY MESSAGES

- Iron deficiency anemia causes oxidative stress and DNA damage in women of childbearing age.
- Hepcidin plays a significant role in iron homeostasis.
- Oxidative stress and DNA damage are reversible with oral iron therapy.

Leukocyte separation from venous blood was performed, followed by the Comet test to assess DNA damage. The statistical analysis of DNA damage characteristics included head length (HL), tail length (TL), head intensity (HI), and tail intensity (TI). The applicable methods are outlined below.

Blood Sampling

Leukocyte Isolation from Blood Samples

Peripheral venous blood was collected in the morning after an overnight fast using a 10-mL vacutainer tube containing K3EDTA (Vacusera, Türkiye). Histopaque-1077 was employed for lymphocyte separation. An equal volume of phosphate-buffered saline was used to dilute the blood, which was then transferred to a Leucosep tube (Greiner Bio-One, Austria). Centrifugation was performed at room temperature for 15 minutes at 800 g. The buffy coat was subsequently removed and washed three times with phosphate-buffered saline (PBS).

Serum Isolation from Blood Samples

Eight milliliters of peripheral venous blood were collected in the morning from subjects who had fasted overnight. Blood samples were obtained using gel tubes (Vacusera, Türkiye) equipped with vacuum seals. Centrifugation was performed for six minutes at room temperature at 7260 rpm to separate the samples from cellular fragments. The serum samples were then frozen at 80°C until analysis.

TOS Measurement

In an acidic environment, the oxidants in the sample convert the ferrous ion-chelator complex into ferric ions, which then interact with the chromogen to increase absorbance. The spectrophotometric absorbance of the sample increases proportionally with the concentration of oxidant molecules. A commercial kit (Rel Assay Diagnostic, Gaziantep, Türkiye) was used to measure TOS in serum homogenates after the study. Oxidants (e.g., lipids, proteins, etc.) and color intensity were measured spectrophotometrically using an Enzyme-Linked Immunosorbent Assay (ELISA) reader set to 492 nm. The range of TOS measured by ELISA was 0.2–80 µmol H_2O_2 equivalent/L. The intra-assay and inter-assay coefficients of

variation (CV) were 2.8% and 3.3%, respectively. Data were expressed as µmol H_2O_2 equivalent/L.

TAS Measurement

The sample's antioxidants reduce the blue-green 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical to colorless reduced ABTS, which forms the basis of the measurement. Antioxidant concentration is directly proportional to absorbance. TAS was measured spectrophotometrically using a 405 nm ELISA reader (Rel Assay Diagnostic, Gaziantep, Türkiye). The TAS ELISA test range was 0.1–3.5 Trolox equivalent/L. The coefficients of variation within and between tests were 3.9% and 3.2%, respectively, with results expressed in mmol Trolox equivalent/L.

OSI Measurement

The oxidative stress index, an indicator of oxidative stress levels, was calculated using the following formula:

OSI (arbitrary unit) = [TOS (µmol H_2O_2 equivalent/L) / TAS (mmol Trolox equivalent/L)]×100.

DNA Damage Analysis with Comet Assay

Five milliliters of anticoagulated blood from the experimental group was diluted 1:1 with a phosphate buffer in a tube. A tube containing 3 milliliters of Ficoll-1077 and 10 milliliters of diluted blood was centrifuged at 400 g for 20 minutes. Following centrifugation, the pellet was washed 2–3 times with 1 ml of RPMI, counted using a hemocytometer, and adjusted to 2×10^4 cells per 100 µL. At 37°C, 80 µL of the lymphocyte suspension was resuspended in 100 µL of "low-melting" agarose (LMA, BioShop, Canada) prepared in 0.5% Ca^{+2} and magnesium chloride-free PBS. The low-melting agarose (LMA) + cell mixture was thinly spread on a slide pre-coated with 1% "normal melting" agarose and frozen for 30 minutes. A third layer of 70 µL of 0.5% LMA was applied to the slide and chilled for 10 minutes. The slide was then incubated in a cold lysis binding solution at pH 10 for 60 minutes at 40°C to remove cellular proteins. After lysis, the slides were subjected to horizontal gel electrophoresis (BIO-RAD, California, USA) in freshly prepared alkaline electrophoresis buffer for 30 minutes. Electrophoresis was performed at 25 V and 300 mA for 30 minutes at the same temperature. To neutralize the alkaline environment and detergents, the slides were washed three times with 0.4 M Tris-HCl at pH 7.5 and 4°C, with each lasting five minutes. Following neutralization, the slides were prepared for examination under a fluorescence microscope and stained with 60 µL of ethidium bromide at a concentration of 2 µL/mL. DNA damage was assessed using the "Comet Assay IV System (AutoComet)" software, that measured parameters such as HL (µm), TL (µm), HI (% H-DNA in head), and TI (% T-DNA in tail).

Hepcidin Measurement

Hepcidin concentration was measured using an ELISA kit (E-EL-H6013, Elabscience Biotechnology Co., Ltd., Wuhan, China). The assay range for hepcidin levels was 62.50–4000 pg/mL, with a sensitivity of 37.50 pg/mL. The intra-assay and inter-assay coefficients of variation were 4.11% and 5.08%, respectively. The procedure was performed according to the manufacturer's instructions, with results expressed in pg/mL.

Other Measurements

Serum iron and total iron-binding capacity (TIBC) were measured using a Cobas C702 autoanalyzer. Ferritin levels were determined with a Cobas E801 autoanalyzer (Roche Diagnostics, Germany), while hemogram variables were assessed using a BC-6800 hematology analyzer (Mindray, China).

Statistical Analysis

Descriptive statistics included the median, minimum, maximum, frequency, and standard deviation. The Kolmogorov-Smirnov test was used to assess the distribution of variables. Quantitative independent data were analyzed using the Mann-Whitney U test and independent sample t-test. For dependent quantitative data, the Wilcoxon test and paired sample t-test were applied. We used the Spearman correlation test for statistical analysis, which was conducted using licensed IBM SPSS (Statistical Package for the Social Sciences) Statistics for Windows, version 28.0 (IBM Corp., Armonk, New York, United States). A p-value of less than 0.05 was considered statistically significant at the 95% confidence level.

RESULTS

In the patient group, there was a significant decrease in hemoglobin (Hb), hematocrit (HCT), mean cell volume (MCV), red blood cell count (RBC), ferritin, serum iron, and hepcidin levels ($p<0.001$). Conversely, serum total iron-binding capacity and red blood cell distribution width (RDW) were significantly increased in the patient group ($p<0.001$). The OSI was significantly higher ($p=0.01$), and TAS was significantly lower ($p=0.02$) in the patient group. However, TOS values did not differ significantly between the two groups ($p>0.05$). The values of HL, TL, and TI were significantly higher in the patient group ($p=0.02$, $p<0.001$, and $p<0.001$, respectively). In contrast, HI was significantly reduced in the patient group ($p<0.001$) (Table 1).

Post-treatment analysis showed no significant change in body mass index (BMI) values compared to pre-treatment levels ($p>0.05$). Post-treatment, the values of hemoglobin, hematocrit, mean corpuscular volume, red blood cells, ferritin, serum iron, and hepcidin showed a significant increase compared to pre-treatment levels ($p<0.001$).

Table 1. Intergroup analysis of demographic and laboratory findings

Parameter	Control group	Patient group	p
Age† (years)	31.5 (27–40.8)	37 (27–42)	0.57*
BMI† (kg/m^2)	23.7 (21.3–27.4)	25.2 (22.7–29)	0.11*
Hb† (g/dL)	13.8 (13.1–14.5)	10.6 (9.9–11.2)	**<0.001***
HCT‡ (%)	40.5±2.2	32.9±2.7	**<0.001****
MCV† (fL)	87.9 (85.9–90)	76 (67.2–81.7)	**<0.001***
RBC‡ (M/uL)	4.65±0.25	4.41±0.36	**0.009****
RDW† (%)	13.1 (12.7–13.4)	16.6 (15.4–17.3)	**<0.001***
Ferritin† (µg/L)	38.6 (32.2–51.6)	5.4 (3.6–7.2)	**<0.001***
Serum iron† (µg/dL)	88.5 (70.8–98.8)	29 (24–38)	**<0.001***
Serum TIBC‡ (µg/dL)	323.1±29.8	411.4±51.9	**<0.001****
Hepcidin‡ (pg/mL)	2300±900	800±600	**<0.001****
TAS‡ (mmol trolox equivalent/L)	1.03±0.27	0.90±0.24	**0.02****
TOS† (µmol H_2O_2 equivalent/L)	13.3 (9–16.1)	14.9 (12.7–17.2)	0.10*
OSI† (arbitrary unit, A.U)	1.5 (0.7–1.7)	1.7 (1.4–2.1)	**0.01***
Head length‡ (µm)	31.1±1.8	32.2±2.5	**0.02****
Tail length† (µm)	28.9 (24.2–31.7)	38.4 (30.6–44.2)	**<0.001***
Head intensity‡ (%)	80.2±10.8	68.6±12.2	**<0.001****
Tail intensity‡ (%)	19.8±10.8	31.4±12.2	**<0.001****

†: Values are given as median (Q1 and Q3 values in parentheses); ‡: Values are given as mean±standard deviation; *: Mann-Whitney U test; **: Independent-samples t-test. BMI: Body mass index; Hb: Hemoglobin; HCT: Hematocrit; MCV: Mean cell volume; RBC: Red blood cell; RDW: Red blood cell distribution width; TIBC: Total iron binding capacity; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

Conversely, both TIBC and RDW values significantly decreased after therapy compared to their pre-treatment levels (p<0.001). Additionally, the TAS value was substantially higher post-treatment than pre-treatment (p<0.001), indicating a marked improvement. There was a significant decrease in TOS and OSI readings after therapy compared to pre-treatment levels (p=0.02 and p=0.002, respectively). The post-treatment HI value was significantly higher than the pre-treatment value (p<0.001). Additionally, relative to pre-treatment values, the post-treatment HL, TL, and TI values showed substantial reductions (p=0.005, p<0.001, and p<0.001, respectively) (Table 2).

The levels of Hb and ferritin did not show a significant correlation with TAS and TOS outcomes (both p>0.05). However, the OSI value exhibited a significant negative correlation with Hb, ferritin, and serum iron levels (r=-0.27, p=0.01; r=-0.27, p=0.01; r=-0.23, p=0.04, respectively). No significant correlation was observed between the TAS value and the HL value (p>0.05). The TAS value demonstrated a significant negative correlation with both TL (r=-0.27, p=0.01) and TI (r=-0.28, p=0.01). A strong positive correlation was identified between TAS and HI values (r=0.28; p=0.01). In contrast, the TOS value did not exhibit any significant correlation with HL, TL, HI, or TI values (p>0.05). The OSI score showed a significant negative correlation with the HI value (r=-0.24; p=0.03) and positive correlations with TL and TI values (r=0.30, p=0.007; r=0.24, p=0.03, respectively). There was no discernible correlation between OSI and HL values (p>0.05) (Table 3). A significant positive correlation was observed between hepsidin levels and both Hb and serum ferritin values (r=0.63, p=0.00; r=0.81, p=0.00, respectively).

DISCUSSION

Iron deficiency can impair the proliferation and development of hematopoietic cells, placing stress on the hematopoietic system. This phenomenon is linked to clonality and complex clinical conditions.[11] In IDA, the functionality of iron-containing antioxidants is compromised, leading to elevated ROS. As a result, erythrocyte loss is accelerated, culminating in eryptosis.[12]

In female patients with IDA, our findings indicated a downregulation of antioxidant stress, as well as increased oxidative stress and DNA damage.[13] TOS levels were also

Table 2. Post–treatment analysis in the patient group of demographic and laboratory findings

Parameter	Pre-treatment	Post-treatment	p
BMI† (kg/m^2)	25.2 (22.7–29)	25.4 (22.3–29)	0.58***
Hb† (g/dL)	10.6 (9.9–11.2)	12.8 (12.2–13.5)	**<0.001*** **
HCT‡ (%)	32.9±2.7	39.1±2.1	**<0.001****
MCV† (fL)	76 (67.2–81.7)	83.5 (80.1–86.9)	**<0.001*** **
RBC‡ (M/uL)	4.41±0.36	4.72±0.34	**<0.001****
RDW† (%)	16.6 (15.4–17.3)	15.3 (12.4–20.3)	**<0.001*** **
Ferritin† (µg/L)	5.4 (3.6–7.2)	27 (23–41)	**<0.001*** **
Serum iron† (µg/dL)	29 (24–38)	70 (58–81)	**<0.001*** **
Serum TIBC‡ (µg/dL)	411.4±51.9	305.6±56.8	**<0.001****
Hepcidin‡ (pg/mL)	800±600	1480±590	**<0.001****
TAS‡ (mmol trolox equivalent/L)	0.90±0.24	1.20±0.35	**<0.001****
TOS† (µmol H_2O_2 equivalent/L)	14.9 (12.7–17.2)	12.4 (10.5–16.4)	**0.02*** **
OSI† (arbitrary unit, A.U.)	1.7 (1.4–2.1)	1.1 (0.8–1.6)	**0.002*** **
Head length‡ (µm)	32.2±2.5	30.8±1.6	**0.005****
Tail length† (µm)	38.4 (30.6–44.2)	25.5 (22–30.8)	**<0.001*** **
Head intensity‡ (%)	68.6±12.2	84.4±8.2	**<0.001****
Tail intensity‡ (%)	31.4±12.2	15.6±8.2	**<0.001****

†: Values are given as median (Q1 and Q3 values in parentheses); ‡: Values are given as mean±standard deviation; ***: Wilcoxon test; ****: Paired-samples t-test. BMI: Body mass index; Hb: Hemoglobin; HCT: Hematocrit; MCV: Mean cell volume; RBC: Red blood cell; RDW: Red blood cell distribution width; TIBC: Total iron binding capacity; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

Table 3. Correlations between oxidative stress indicators and other variables before treatment

	TAS		TOS		OSI	
	r	p	r	p	r	p
Hb (g/dL)	0.17	0.12	-0.17	0.13	-0.27	**0.01**
Ferritin (µg/L)	0.17	0.13	-0.13	0.24	-0.27	**0.01**
Serum iron (µg/dL)	0.15	0.18	-0.14	0.22	-0.23	**0.04**
Head length (µm)	0.05	0.66	0.22	0.05	0.16	0.16
Tail length (µm)	-0.27	**0.01**	0.14	0.22	0.30	**0.007**
Head intensity (%)	0.28	**0.01**	-0.09	0.41	-0.24	**0.03**
Tail intensity (%)	-0.28	**0.01**	0.09	0.41	0.24	**0.03**

Spearman Correlation. TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index; Hb: Hemoglobin.

elevated.[14] Our study further demonstrated reduced antioxidant levels and increased DNA damage, as assessed by the Comet test. However, while TOS and TAS levels did not change significantly, neither did the correlation between TOS and Hb, ferritin, or serum iron. This outcome may be attributed to the exclusion of severely anemic individuals from our research. However, we identified a negative correlation between the OSI score and both Hb and ferritin levels (Table 3). Our data suggest that antioxidant levels play a more critical role in determining OSI levels in individuals who are not severely anemic.

Conflicting results have been reported in both animal and human studies regarding the effects of iron treatment on oxidative stress markers in IDA.[15,16] Additionally, the threshold of iron that induces oxidative DNA damage is

currently unclear.[17] A 25 mg dose of ferrous sulfate has been shown to increase ROS production.16 In another study involving pediatric patients, a daily oral dose of 10 mg ferric ammonium citrate was found to increase antioxidant capacity and decrease DNA damage.[18] Our investigation demonstrated that oral iron supplementation significantly elevated TAS values post-treatment compared to pre-treatment measurements. Additionally, post-treatment TOS and OSI readings showed a significant reduction compared to pre-treatment levels. This indicates that oxidative stress results from IDA in the body, and while iron is an oxidant, oxidative stress diminishes and antioxidant levels increase with iron replacement. Our investigation has shown that oral iron replenishment significantly elevated the TAS value post-treatment compared to the pre-treatment measurement. Nonetheless, the exact quantity of iron required to mitigate oxidative levels in the body, as suggested by the literature, could not be determined, representing a limitation of our study. In this regard, we suggest that future research with shorter intervals and extended durations is necessary for more accurate measurement of these values.

One study using the Comet assay in children found that iron deficiency caused less DNA damage, while another indicated that iron deficiency increased DNA damage, which improved with iron treatment.[18,19] Our findings suggest that DNA damage may be exacerbated by IDA and alleviated by oral iron therapy. Discrepancies in other studies may be attributed to limited sample sizes or the age group studied, which often corresponds to a developmental stage characterized by significant growth and metabolic activity. This study is the first to evaluate the effect of oral iron therapy on DNA damage using Comet analysis in reproductive-age women with IDA. Analysis of the relationship between oxidative variables and DNA damage data derived from the Comet assay revealed a strong negative correlation between TAS values and both TL and TI values (Table 3). Conversely, a significant positive correlation was observed between TAS values and HI values (Table 3), suggesting that DNA damage increases as total antioxidant levels in the body decrease. No link was identified between TOS values and DNA damage parameters derived from the Comet assay in our data. However, there was a significant inverse association between OSI scores and head intensity values. Additionally, the notable negative correlation between OSI scores and HI values (Table 3), along with the substantial positive correlation between OSI scores and both TL and TI values (Table 3), highlights the influence of antioxidant activity on DNA damage. This finding suggests that reduced antioxidant levels in the body contribute to elevated OSI. The significant improvements in DNA damage metrics observed through the Comet assay following oral iron supplementation indicate that DNA damage in individuals with IDA is reversible.

A review of the literature shows that hepcidin levels are typically reduced in individuals with IDA and increase following iron supplementation.[3] Our findings align with this, as hepcidin levels were elevated in cases of iron repletion.[20] Furthermore, a substantial positive correlation was noted between hepcidin levels and both Hb and ferritin concentrations. Upon analyzing the association data, we encountered discrepancies across the research in the literature.[21] These discrepancies can be attributed to variations in sample selection, treatment modalities, patient demographics, application techniques, iron formulations, geographical factors, and the lack of a unified standard testing apparatus. Additionally, some studies have explored the association between exercise and hepcidin levels.[5] Exercise may be considered a limitation in our study. Consequently, supplementation with approximately 100 mg of elemental iron elevated hepcidin levels. Our research indicates that hepcidin plays a crucial role in IDA.

Iron deficiency can disrupt the proliferation and differentiation of hematopoietic cells by creating stress on the hematopoietic system. This may contribute to clonality and complex clinical conditions.[11] If IDA is not effectively treated, it can progress to clonality and myelodysplastic syndrome. IDA is considered a global public health problem, impacting the health, social, and economic processes of individuals and societies in both developed and underdeveloped regions. Early detection and treatment of anemia in women can prevent conditions associated with severe morbidity and mortality. This is particularly important for women of childbearing age, as untreated anemia increases the risks of premature birth, low birth weight, perinatal complications, maternal mortality, and complications during childbirth.

CONCLUSION

In conclusion, this study reaffirms that hepcidin plays a crucial role in iron regulation, and we demonstrated that enhanced oxidative stress and DNA damage in IDA can be ameliorated after just one month of oral iron therapy. It is reassuring that both oxidative stress and DNA damage improved with oral iron treatment. Oral iron supplementation is recommended for women of reproductive age to restore the compromised antioxidant defense system. If IDA is not effectively treated, it may lead to clonality and myelodysplastic syndrome. Early detection and treatment of anemia in women can prevent illnesses associated with significant morbidity and mortality. This is particularly important for reproductive-age women, as untreated anemia increases the risks of preterm delivery, low birth weight, perinatal complications, and maternal mortality, and complications during childbirth.

Ethics Committee Approval: The Pamukkale University Non-interventional Clinical Research Ethics Committee granted approval for this study (date: 03.11.2020, number: 60116787-020/66544).

Author Contributions: Concept – GAC, MNB, ACD; Design – GAC, EKT, MNB; Supervision – GAC, IHA; Resource – GAC, MNB; Materials – GAC, MNB; Data Collection and/or Processing – GAC, MNB, MTA, FA; Analysis and/or Interpretation – GAC, MNB; Literature Search – GAC, MNB; Writing – GAC, MNB; Critical Reviews – GAC, IHA, ACD, EKT.

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