SERUM-ASSOCIATED IMPAIRMENT OF PHAGOCYTIC ACTIVITY AGAINST *KLEBSIELLA PNEUMONIAE* BY MONOCYTE-DERIVED MACROPHAGES FROM HEMOGLOBIN E/β-THALASSEMIA SUBJECTS

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Abstract. Bacterial infection is one of the leading causes of death in thalassemia patients. This study aimed to quantify macrophage phagocytic activity against *Klebsiella pneumoniae* in moderate-to-severe HbE/β-thalassemia subjects and investigate its correlation with ferritin and uric acid levels. Understanding this is crucial to understanding their susceptibility to infection. This cross-sectional study comprised hemoglobin E (HbE)/beta-thalassemia subjects (n = 19) and healthy participants (n = 7) as controls. Exclusion criteria were cancer, current infection, diabetes, or receiving immunosuppressive treatment. HbE/beta-thalassemia subjects had a median serum ferritin and uric acid level of 1,221 ng/ml and 6.0 mg/dl respectively, significantly higher than that of 119.6 ng/ml and 4.4 mg/dl respectively for healthy controls (p-value = 0.0002 and 0.0259 respectively). To evaluate the phagocytic activity of monocyte-derived macrophages (MDM), the percent heated-inactivated carboxyfluorescein succinimidyl ester-stained *Klebsiella pneumoniae*-ingested MDM was measured by flow cytometry. The MDM phagocytic activity of HbE/β-thalassemia subjects and healthy controls is not significantly different (19.63 *vs* 8.77 %, respectively; p-value = 0.063); however, following the addition of autologous serum, the increase in phagocytic activity was significantly smaller in the former relative to the latter group (Δ36.97 *vs* Δ63.46 % respectively; p-value <0.01). A linear regression analysis showed no correlation of ferritin or uric acid level with MDM phagocytic activity of HbE/β-thalassemia subjects. Our results suggest that HbE/β-thalassemia subjects have reduced phagocytic activity against *K. pneumoniae* compared to healthy controls, possibly contributing to their increased susceptibility to infection. Future studies should explore other factors affecting phagocytic activity in β-thalassemia.

Keywords: *Klebsiella pneumoniae*, ferritin, HbE/β-thalassemia, phagocytosis, uric acid

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INTRODUCTION

β-Thalassemia is a prevalent genetic disorder in Southeast Asia, including Thailand, where the presence of a high frequency of hemoglobin E leads to HbE/β-thalassemia genotype, which accounts for half of the severe β-thalassemia cases in the region and worldwide (Olivieri *et al*, 2010). Hemoglobin E is caused by a mutation in codon 26 (GAG >AAG) of the *HBB* gene, resulting in an alternative splice site and reduction in β^E-mRNA (Orkin *et al*, 1982).

Infection is a significant cause of morbidity and mortality in β-thalassemia patients, second only to heart failure (Borgna-Pignatti *et al*, 2004). Individuals with HbE/β-thalassemia are particularly susceptible to bacterial, viral and fungal infections, with bacterial septicemia posing a significant threat (Teawtrakul *et al*, 2015). Even among unsplenectomized HbE/β-thalassemia subjects, the higher prevalence of severe infections indicates that their increased susceptibility to infection is not solely due to splenectomy-related effects. In a study conducted in Thailand involving 211 thalassemia patients (60% with HbE/β-thalassemia), severe infections were observed in 11 (5%) patients, among whom five have not undergone splenectomy (Teawtrakul *et al*, 2015). *Klebsiella* and *Burkholderia* infections were the most common among both splenectomized and unsplenectomized patients. This underscores a critical gap in our understanding of the immune dysfunctions in thalassemia patients.

Phagocytosis, a key immune defense mechanism, has been reported to be impaired in β-thalassemia. Several studies of β-thalassemia patients have shown reduced phagocytic activity in neutrophils (Quintiliani *et al*, 1983; van Asbeck *et al*, 1984; Matzner *et al*, 1993; Srinoun *et al*, 2017) and in monocytes (Wanachiwanawin *et al*, 1993; Kuno *et al*, 2019). In contrast, other studies have demonstrated

increased phagocytic activity (Bassaris *et al*, 1982; Skoutelis *et al*, 1984; Sternbach *et al*, 1987; Wiener *et al*, 1996; Wiener *et al*, 1999), especially in monocytes co-cultured with β-thalassemia red cells. These inconsistencies in findings could be partly explained by differences in culture conditions and different pathogens used in the experiments.

Furthermore, early studies suggested a connection between phagocytic defects and (unknown) factors in β-thalassemia sera (Bassaris *et al*, 1982; Skoutelis *et al*, 1984). Iron overload, common in thalassemia patients receiving frequent blood transfusions, is linked to impaired phagocytic function (Sternbach *et al*, 1987; van Asbeck *et al*, 1984). Additionally, hyperuricemia, another condition frequently observed in thalassemia, is associated with oxidative stress and may impact phagocytic activity through mechanisms related to iron overload (Vassalle *et al*, 2018; Vlachaki *et al*, 2011) or NLRP3 (a member of the leucine-rich repeat-containing proteins (NLRP) family) inflammasome activation (Kodama *et al*, 2009; Seifi *et al*, 2024).

Despite numerous studies on phagocytic function in β-thalassemia, there is a lack of research simulating the *in vivo* environment. We evaluated the phagocytic function of monocyte-derived macrophages from HbE/β-thalassemia subjects against *Klebsiella* bacteria, the leading cause of severe infection in Thailand. We also investigated the impact of thalassemia serum on phagocytosis and its association with iron and uric acid levels. By better understanding the immunodeficiency mechanisms in HbE/β-thalassemia patients in response to *Klebsiella* infection, we aim to provide new insights into potential mechanistic treatments and preventive strategies for infections.

MATERIALS AND METHODS

Participants

We conducted a cross-sectional study of recruited subjects >20 years of age with moderate to severe, transfusion-dependent HbE/β-thalassemia who had been followed up at Naresuan University Hospital, Phitsanulok Province, Thailand for at least 6 months before recruitment to ensure complete documentation of their previous medical history. Four splenectomized and fifteen non-splenectomized patients were recruited. The study was conducted from October 2020 to September 2021. Exclusion criteria were

cancer, current infection with body temperature >37.5 °C, diabetes, or on prescribed immunosuppressive drugs. Age-matched healthy controls with normal complete blood count were included for comparison.

Data collection

All participants underwent blood collection to assess blood indices, liver and renal function, uric acid, ferritin, and C-reactive protein levels, and phagocytic activity using their monocyte-derived macrophages (MDMs). To assess MDM phagocytic activity the following procedures were performed:

i. *MDM generation:* Peripheral blood mononuclear cells (PBMCs) were isolated from 20 ml of EDTA blood from HbE/β-thalassemia and normal participants. PBMCs (10-20×10^6 cells) in RF10 culture medium [RPMI medium (Thermo Fisher Scientific, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Paisley, UK)] were incubated at 37 °C in an atmosphere containing 5% CO_2 for 1.5 hours. Non-adherent cells were removed by washing twice with phosphate-buffered saline pH 7.4 (PBS) and adherent cells subsequently were detached using a rubber policeman. Detached cells were stained with trypan blue and counted under a light microscope, 100× magnification. Monocytes were identified by flow cytometry after treatment with anti-CD14-PerCP-Cy5.5 antibodies (BD Biosciences, Franklin Lakes, NJ). Monocytes then were cultured in RF10 medium supplemented with 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Cranbury, NJ) and incubated at 37 °C as described above for seven days. Fresh RF10 medium and GM-CSF were added on days 4 and 6, and 50 ng/ml recombinant human interferon-gamma (IFN-γ) (PeproTech, Cranbury, NJ) were added on Day 6. On Day 7, adherent cells were trypsinized, centrifuged at 400g at 4 °C for 5 minutes, and resuspended in 200 μl of RF10 culture medium. MDMs were identified by flow cytometry after treatment with anti-CD16-PE (BD Biosciences,

Franklin Lakes, NJ) and anti-CD80-APC (BioLegend, San Diego, CA) antibodies (Fig 1).

ii. *Bacteria preparation:* One ml aliquot (1×10^8 CFU) of heat-killed (65 °C for 15 minutes) *Klebsiella pneumoniae* (kindly prepared by Assoc Prof Dr Pannika Ritvirool,

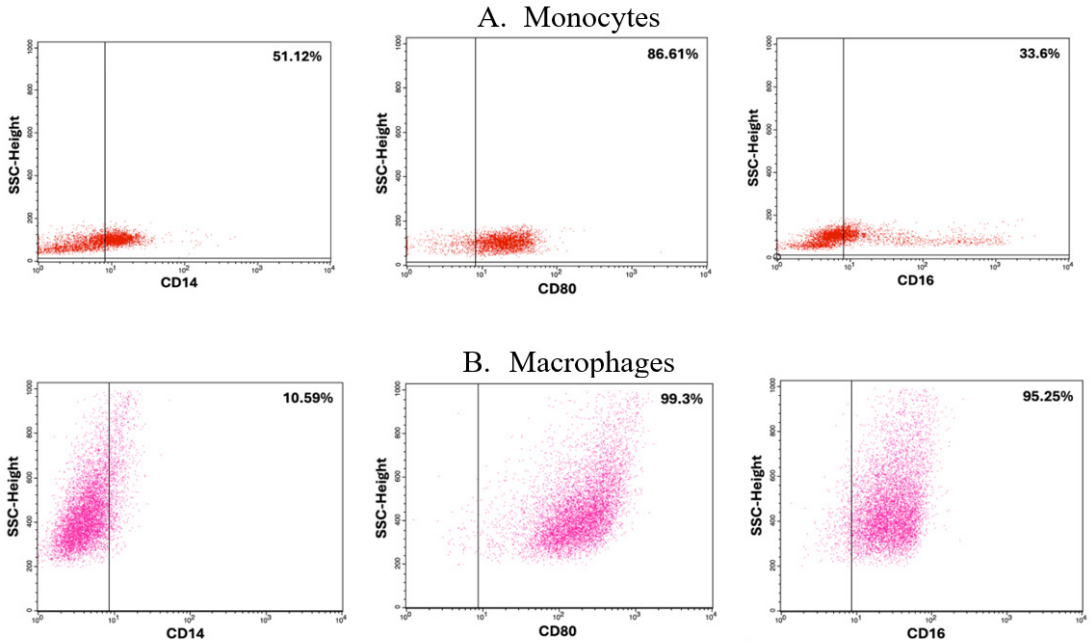

Fig 1 - Identification of serum monocytes and macrophages by cell cytometry analysis

A: Monocyte surface has a high level of CD14 and low levels of CD16 and CD80.

B: Monocyte-derived macrophage (MDM) surface is devoid of CD14 but contains CD16 and CD80. CD14 and CD80 are identified with antibodies conjugated with Allophycocyanin (APC) and CD16 with antibodies conjugated with Phycoerythrin (PE). The X-axis represents the level of tagged antibodies and Y-axis the level of side scatter (SSC), which reflects the internal complexity or granularity of the cell. The combination of antibody expression and SSC allows different cell populations to be distinguished and quantified.

Faculty of Medical Bioscience, Naresuan University) was centrifuged at 4,000g for 5 minutes. The pellet was treated with 1 ml of 2× carboxyfluorescein succinimidyl ester (CFSE) (BioLegend, San Diego, CA), incubated in the dark at 37 °C for 30 minutes, then washed three times with PBS, resuspended in 1 ml of PBS, and stored at 4 °C until used.

iii. *Phagocytic activity assessment:* MDMs were mixed with CFSE-stained heat-killed *K. pneumoniae* (1:1000) and incubated at 37 °C as described above for 3 hours, with mixing every hour. Nonphagocytized *K. pneumoniae* were removed with 300 µl of PBS and the remaining cells were centrifuged at 400g for 5 minutes at 4 °C and resuspended in PBS. MDMs were treated with 5 µg/ml anti-CD16-PE (BD Biosciences, Franklin Lakes, NJ) and anti-CD80-APC (BioLegend, San Diego, CA) antibodies for 20 minutes at 4 °C and subjected to flow cytometry. Phagocytic activity of MDMs was determined by quantifying cells double-positive for CD80-APC and CFSE using FlowJo software (Tree Star, Portland, OR) and presented as percent MDMs counted.

Three comparisons of phagocytic activity were performed: Comparison 1, baseline values between HbE/β-thalassemia subjects and healthy controls; Comparison 2, values upon addition of autologous serum between the two groups; Comparison 3, the effect of cross-incubation, in which control MDMs were incubated with heat-inactivated *K. pneumoniae* either in the presence of autologous serum or serum of three randomly chosen HbE/β-thalassemia subjects.

Data analysis

Demographic data were presented as medians. Comparison of phagocytic activity between HbE/β-thalassemia subjects and healthy controls was determined using a Mann-Whitney U test. In the cross-incubation experiments, phagocytic activity of control MDMs in autologous sera was compared to the median value in three HbE/β-thalassemia sera using a Wilcoxon matched-pairs signed-rank test. The relationships between laboratory parameters with phagocytic activity were

determined using univariate linear regression analysis. A *p*-value of <0.05 is considered statistically significant. All statistical analyses were performed using a Statistical Package for Social Sciences (SPSS) software version 17.0 (IBM Corp, Armonk, NY).

Ethical approval

The study protocols were approved by the Naresuan University Institutional Review Board (NU-IRB), approval no. P3-0135/2563. Prior written consent was obtained from each participant.

RESULTS

HbE/β-thalassemia subjects' demographic and laboratory data

The median age of the HbE/β-thalassemia group (*n* = 19) was 39 years (range 23-72 years) while that of the healthy control group (*n* = 7) was 31 years (range 24-49 years) (not significantly different between the two groups) (Table 1). The gender ratio between the two groups was comparable. The median hemoglobin (Hb) level (7.3 g/dl), hematocrit (Hct) (23.3%) and mean corpuscular volume (MCV) (66 fl) of the HbE/β-thalassemia group are significantly lower than those of the healthy control group (*p*-values = 0.0001, 0.0001, and 0.0020, respectively), while median white blood cell count (WBC) (8,560 cell/mm^3) is marginally higher (*p*-value = 0.0528), and median uric acid (6.0 mg/dl) and ferritin levels (1,221 ng/ml) are significantly higher (*p*-values = 0.0259 and 0.0002, respectively); however, there is no significant difference in median platelet count between the two groups (244,000 *vs* 252,000 cell/mm^3; *p* = 0.7505) (Table 1).

MDM phagocytic activity

Fig 2 shows typical results of the flow cytometry method for determining MDM phagocytic activity of heat-inactivated *K. pneumoniae*. Before serum addition, the median MDM phagocytic activity is not significantly different between HbE/β-thalassemia and healthy control groups (Table 2). However, after the addition of autologous serum, the median phagocytic activity in the healthy control group is significantly higher than that of the HbE/β-thalassemia group (81.12% *vs* 67.14%, *p*-value = 0.0151). The difference in median phagocytic activity before and after the addition of autologous serum is also significantly higher in the healthy control group than in the HbE/β-thalassemia group (*p*-value <0.01).

As it was reported that thalassemia serum contains factors negatively impacting phagocytosis (Bassaris *et al*, 1982; Skoutelis *et al*, 1984), a cross-serum experiment was carried out. MDMs from five healthy subjects were incubated with serum from three HbE/β- thalassemia subjects. As expected, the median phagocytic activity post-addition of autologous serum was higher than that pre-addition (32.26% *vs* 12.93%, *p*-value = 0.043) (Table 3). However, after incubation with serum from

Table 1

Demographic and laboratory data of HbE/β-thalassemia subjects and healthy participants

Demographic and laboratory parameter	HbE/β-thalassemia subject (n = 19)	Healthy participant (n = 7)	p-value*
Gender, number			0.7798
Male	7	3	
Female	12	4	
Age (years), median	39	31	0.5434
Hb (g/dl), median	7.3	12.8	0.0001
Hct (%), median	23.3	38.3	0.0001
MCV (fl), median	66	85.2	0.0020
WBC (cell/mm^3), median	8,560	5,850	0.0528
Platelets (cell/mm^3), median	244,000	252,000	0.7505
Uric acid (mg/dl), median	6.0	4.4	0.0259
Ferritin (ng/ml), median	1,221	119.6	0.0002
Blood transfusion (unit/year), median	20	N/A	

*Significantly different when p-value <0.05.

Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume; N/A: not applicable; WBC: white blood cell count

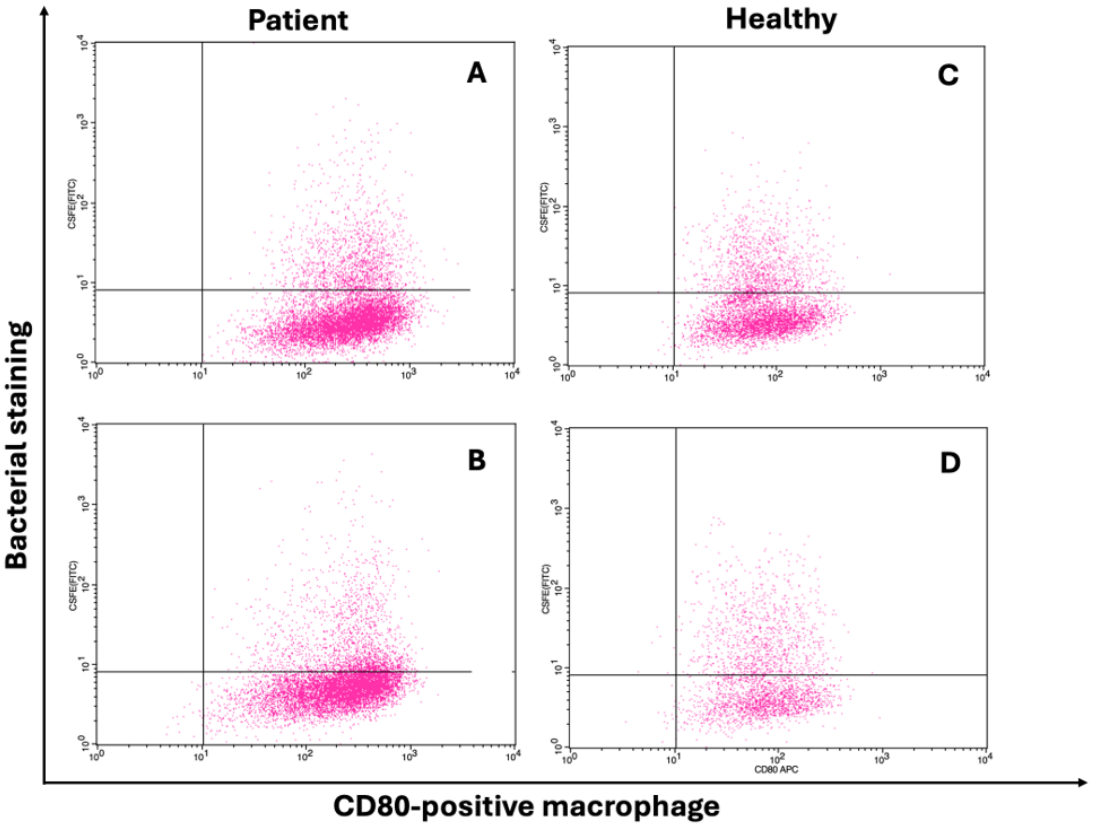

Fig 2 - Phagocytic activity of monocyte-derived macrophages (MDMs) from HbE/β-thalassemia (n = 2) and healthy control (n = 2) subjects using a cell cytometry assay

Phagocytic activity of monocyte-derived macrophages (MDMs) from HbE/β-thalassemia (A: 5.75%; B: 19.14%) and healthy control (C: 23.46%; D: 36.24%) subjects was determined using a cell cytometry assay. MDMs were mixed with heat-killed carboxyfluorescein succinimidyl ester (CFSE)-stained *K. pneumoniae* (1:1000) and incubated at 37 °C under an atmosphere of 5% CO_2 for 3 hours. Phagocytized *K. pneumoniae* cells were removed and prior to cell cytometry assay the mixture was treated with anti-CD80 antibodies conjugated with Allophycocyanin (APC) to label MDM surface. The X-axis represents the level of CD80 and and Y-axis the level of ingested *K. pneumoniae*. Phagocytic activity is defined as percent *K. pneumoniae*-ingested MDMs.

Table 2

Phagocytic activity of monocyte-derived macrophages from HbE/β-thalassemia subjects and healthy participants

Experiment	Phagocytic activity, median percent (IQR)		*p*-value*
	HbE/β-thalassemia subjects (*n* = 19)	Healthy participants (*n* = 7)	
Comparison 1: Before addition of autologous serum	19.63 (11.69-57.58)	8.77 (3.20-36.24)	0.0633
Comparison 2: After addition of autologous serum	67.14 (55.74-74.95)	81.12 (74.28-92.45)	0.0151
Difference between Comparisons 1 and 2	36.97 (9.58-49.17)	63.46 (59.12-69.43)	<0.01

*Significance level at *p*-value <0.05 using Mann-Whitney test.

IQR: interquartile range

Table 3

Phagocytic activity of monocyte-derived macrophages from healthy participants pre- and post-addition of autologous and HbE/β-thalassemia sera

Healthy participant	Phagocytic activity (%)					
	No serum addition	Addition of autologous serum	Addition of HbE/β-thalassemia serum			
			Subject 1	Subject 2	Subject 3	Median
1	13.84	17.35	22.98	18.47	7.12	18.47
2	12.93	58.9	59.76	40.92	26.56	40.92
3	21.32	51.52	23.73	48.22	26.25	26.25
4	4.42	11.68	18.61	18.43	12.06	18.43
5	7.05	32.26	17.27	51.84	29.45	29.45
Median (IQR)	12.93 (7.05-13.84)	32.26 (17.35-51.52)				26.25 (18.47-29.45)

IQR: interquartile range

three individual HbE/β-thalassemia subjects, the median phagocytic activity of the healthy control group was only 26.25%, but not significantly different from that obtained with the addition of autologous serum (p-value = 0.345).

Linear regression analysis of HbE/β-thalassemia MDM phagocytic activity with demographic and laboratory parameters

To determine whether any demographic or laboratory parameters correlated with the phagocytic activity of HbE/β-thalassemia MDMs, univariate linear regression analysis was used. The results showed no statistically significant relationship between the demographic (age) and laboratory parameters with the phagocytic activity after the addition of autologous serum (Table 4), or change in phagocytic activity pre- and post-serum addition of autologous and HbE/β-thalassemia (Table 5).

Table 4

Univariate linear regression analysis of demographic and laboratory parameters and of phagocytic activities* of HbE/β-thalassemia subjects

Demographic and laboratory parameter	Univariate linear regression	
	Beta-coefficient	p-value
Serum ferritin	0.0027882	0.281
Uric acid	3.229659	0.342
Age	0.0760876	0.862
Hemoglobin	3.865624	0.481
Hematocrit	1.784282	0.188
MCV	-1.221447	0.137
WBC count	-0.0000611	0.909
Platelet count	-0.0000165	0.433
Blood transfusion (unit/year)	-0.5952197	0.134

*In presence of autologous serum

MCV: mean corpuscular volume; WBC: white blood cell

Table 5

Univariate linear regression analysis of demographic and laboratory parameters and of normal participants' (n = 5) phagocytic activity pre- and post-addition of autologous serum and that of three HbE/β-thalassemia subjects

Demographic and laboratory parameter	Univariate linear regression	
	Beta-coefficient	p-value
Serum ferritin	0.0042076	0.170
Uric acid	0.7822008	0.850
Age	-0.0557091	0.915
Hemoglobin	7.18375	0.269
Hematocrit	2.609	0.103
MCV	-2.165638	0.021
WBC count	-0.0012075	0.045
Platelet count	-0.0000715	0.001
Transfusion (unit/year)	-0.2811408	0.565

MCV: mean corpuscular volume; WBC: white blood cell

DISCUSSION

Our study demonstrated that MDMs from transfusion-dependent HbE/β-thalassemia subjects (n = 19) exhibited a significantly smaller increase in in vitro phagocytosis of heat-inactivated *K. pneumoniae* in the presence of autologous serum, compared to the MDM phagocytic activity of healthy controls (n = 7). Subsequent cross-incubation experiments (Comparison 3) suggested a potential suppressive effect of HbE/β-thalassemia serum on phagocytic function, although the effect did not reach statistical significance, probably due to the small number of tests.

Sternbach *et al* (1987) observed a reduction in the ability to kill *Candida pseudotropicalis* by mononuclear cells from thalassemia patients who received repeated blood transfusions. Previously, van Asbeck *et al* (1984) reported a reduction in the phagocytic

activity of opsonized *Staphylococcus aureus* by mononuclear cells and neutrophils from patients with iron overload. Although these two studies involved different types of phagocytic cells and different pathogens, the findings highlight the reduction in phagocytic activity of transfusion-dependent β-thalassemia and iron overload subjects, consistent with our study. Our findings expand this notion by demonstrating that the phagocytic activity of heat-inactivated *K. pneumoniae* in HbE/β-thalassemia was also impaired.

Currently, there is a lack of evidence explaining the mechanism(s) of serum-mediated impairment in phagocytosis. Wiener (2003) reported that exposure to β-thalassemia major sera impaired macrophage differentiation, as indicated by a reduction in CD11b/CD18 expression. In contrast to this finding, our study demonstrated that even when using differentiated macrophages from healthy controls, the addition of HbE/β-thalassemia sera resulted in a mild impairment of phagocytic activity (although not reaching a statistically significant level due to the limited number of samples).

We attempted to explain this serum-mediated impairment in phagocytosis by examining correlations with serum ferritin and uric acid levels, as it has been documented that patients with iron overload tend to have impaired phagocytic activity, and hyperuricemia is a good indicator of inflammation and iron overload (Vlachaki *et al*, 2011; Dasgupta *et al*, 2013; Vassalle *et al*, 2018). However, our analysis revealed no discernible correlation between these parameters and impairment in phagocytic activity. This result may be attributed to the limited number of subjects and/or the presence of other (unidentified) serum factors.

The critical limitation of our study was the small number of subjects, potentially impacting the statistical power to detect correlations between laboratory parameters and phagocytic function. Future research should seek to identify other factors, which could negatively impact phagocytosis, and employ a larger cohort of thalassemia subjects to enhance the robustness and generalizability of the findings.

In conclusion, we showed

that autologous sera of HbE/β-thalassemia subjects reduced monocyte-derived macrophage phagocytic function against heat-inactivated *Klebsiella pneumoniae in vitro*, a phenomenon not observed with sera from healthy individuals. It remains to be determined what serum factors are responsible for this impairment in phagocytosis, which may contribute to the increased susceptibility to infection, a major cause of morbidity and mortality in β-thalassemia.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interests.

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