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ORIGINAL RESEARCH

Haematological Indicators of Response to Erythropoietin Therapy in Chronic Renal Failure Patients on Haemodialysis: Impact of Angiotensin-Converting Enzyme rs4343 Gene Polymorphism

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Purpose: This is the first cross-sectional study studying the changes in haematological indicators of the response to recombinant human erythropoietin (rHuEPO) therapy in chronic renal failure (CRF) patients on haemodialysis (HD) stratified according to ACE G2350A (rs4343) gene polymorphism.

Design: An observational cross-sectional study.

Setting: Nephrology department and Biochemistry and molecular biology department, faculty of medicine, Cairo University.

Patients: A total of 256 CRF patients on HD for at least six months (162 male and 103 female) and 160 healthy subjects (122 male and 38 female) were recruited in the current study after signing a consent form. ACE G2350A (rs4343) Insertion/Deletion (I/D) was tested, the association between ACE G2350A (RS4343) gene polymorphisms and patients response to rHuEpo was evaluated.

Results: ACE G2350A (rs4343) I/D was the most prevalent genotype, while I/I genotype was the lowest prevalent among patient or control subjects included in the study. D allele is the most prevalent allele, either among patients or the control group. Hemoglobin (Hb) level in patients with I/I and Deletion/Deletion (D/D) genotype was significantly higher compared to those with I/D genotype (P = 0.012 and P = 0.005, respectively). Serum iron in the I/D genotype was significantly higher than those with either I/I or D/D genotype (P = 0.045 and P = 0.018, respectively). Angiotensin-converting enzyme (ACE) content, total leukocytic count (TLC), and soluble erythropoietin receptor (sEpoR) were independent predictors of Hb level. The ACE gene, TLC, and serum iron were the independent factors that may affect the Haematocrit (Hct) level. ACE G2350A (rs4343) gene polymorphisms may affect the HD patient's responses to rHuEPOs.

Conclusion: In HD patients, screening for ACE G2350A (rs4343) gene polymorphisms before rHuEpo administration may help predict patient response.

Keywords: angiotensin-converting enzyme rs4343 gene polymorphism, hemoglobin, erythropoietin, chronic renal failure, hemodialysis

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Key Message

- Patients with the ACE G2350A (rs4343) I/I or D/D genotype had a higher Hb level than patients with the I/D genotype.

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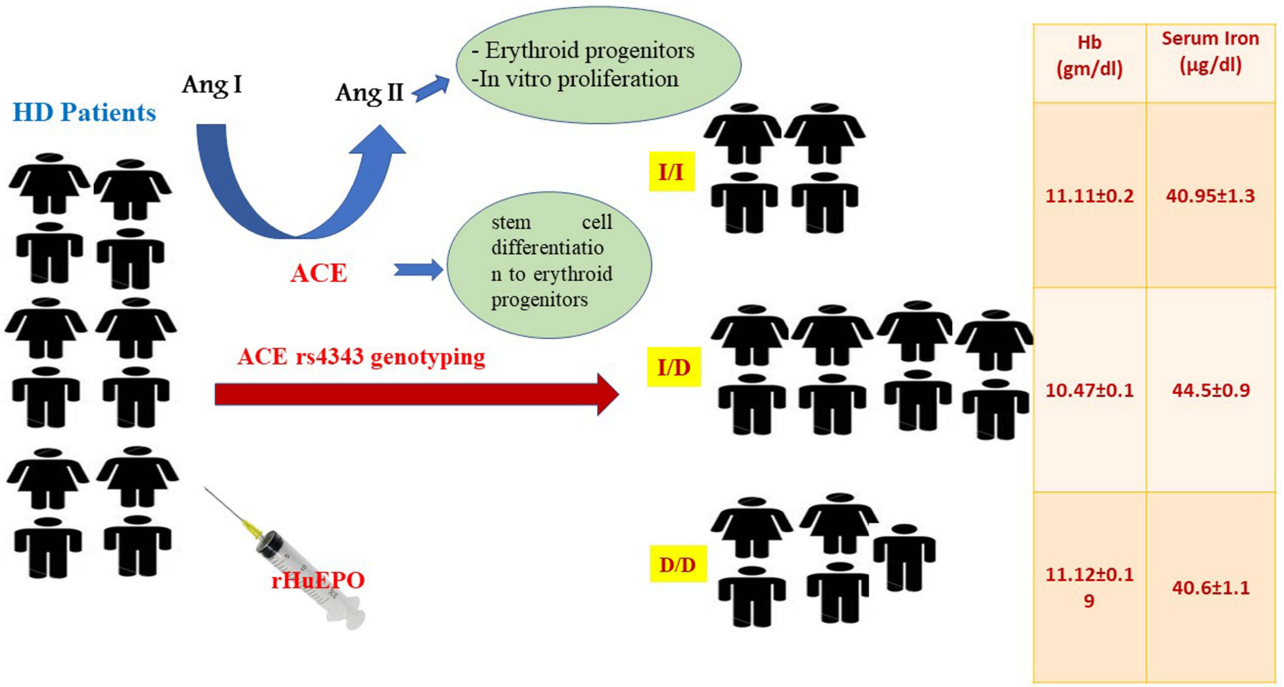
Graphical abstract



- Patients with either the ACE G2350A (rs4343) I/D or D/D genotype had higher iron stores than those with the I/I or D/D genotype.
- ACE protein content, TLC, and soluble erythropoietin receptor (sEpoR) are independent predictors of recombinant human erythropoietin (rHuEPO) response in CRF patients.
- In HD patients, screening for ACE G2350A (rs4343) gene polymorphisms before rHuEpo administration may help predict patient response.

Introduction

Chronic kidney disease (CKD) has a global prevalence of 8–16%, with serious morbidity and mortality.[1] CKD is a direct risk factor for cardiovascular diseases, end-stage renal disease (ESRD)/CRF, and mortality.[2] While replacement therapy with regular dialysis represents a temporary solution, renal transplantation is the permanent solution.[3] Anaemia is one of the most important CRF complications, which develops early and worsens during the long-term progression of the disease.[4] Coresh et al showed the association between lower Hb levels, the severity of anaemia and kidney function reduction.[5] Erythropoietin (Epo), iron therapy, and continuous patient response monitoring provide a good tool for treating CKD-associated anaemia[6] that helps to minimize transfusions and improve CKD patient survival.[7] Although the response to rHuEpo is mostly good, resistance to Epo therapy among these cases ranges from 10% to 20%.[8]

Many factors may affect patients' responses to replacement therapy with rHuEPO, including genetic factors, eg, ACE gene polymorphism that has an important impact on hematopoiesis. ACE gene is located at 17q23. It contains 26 exons and 25 introns.[9] It has several single-nucleotide polymorphisms (SNPs). ACE G2350A (rs4343) SNP is located in exon 17 of the ACE gene and results in silent Thr 776 Thr (NP_000780.1) change. ACE gene SNPs may affect the patient's response to Epo and could be useful genetic markers in assessing the required dose of Epo in such patients.[10] ACE SNPs effect on CKD response to Epo therapy was evaluated with conflicting results. Varagunam et al reported a predictive role for it in determining Epo dosage in continuous ambulatory peritoneal dialysis English patients,[11] while in another study in Korean HD patients, it was found to be associated with Epo resistance.[10] ACE G2350A (RS4343) was selected for the present study based on a genome-wide-analysis study that reported the ACE G2350A (RS4343) is a good predictor of ACE activity[12] due to the absence of wide genomic mapping in Arabian Countries, so our hypothesis that it may affect HD patients' response to rHuEPO.

Although it was investigated concerning other clinical conditions, to the best of our knowledge, none of the international reports studied the effect of ACE G2350A (RS4343) gene polymorphisms on haematological markers of response to rHuEpo in CRF patients on HD. The current study aims to study the effect of ACE G2350A (RS4343) I/D gene polymorphisms on the response to rHuEpo, anaemia biomarkers, ACE content, inflammatory biomarkers, serum Epo and soluble Epo receptor (sEpoR) among CRF patients on HD.

Patients & Methods

Patients and Design

Design

Observational cross-sectional study.

Setting

Nephrology department and Biochemistry and molecular biology department, faculty of medicine, Cairo University.

Patients

Our cross-sectional study enrolled 256 CRF patients on HD for ≥six months receiving rHuEpo therapy. They included 162 males and 103 females and aged 51.3 ± 11.9 years. They were recruited from the nephrology unit, Internal Medicine Department, Cairo University, Cairo, Egypt, from April 2019 to June 2020. Matching 160 normal healthy control subjects were recruited from those accompanying outpatients and comprised 122 males and 38 females ageing 36.1 ± 12.8 years (Table 1). Each participant had a five-minute interview to discuss the current study's objectives and aims before signing the informed consent and enrollment.

Exclusion Criteria

Patients excluded from the study if age <18 years, acute renal failure, non-CKD-related anaemia, recent blood transfusion within the previous three months, a history of hepatitis B (HBV) or C (HCV) or HIV or other active acute or chronic infections, decompensated liver cirrhosis, pregnancy, and malignancy.

Blood Sampling and Investigations

10 mL peripheral venous blood was collected on heparin. The recovered plasma by centrifugation (1000 x g for 10 min at 4 °C) was aliquot stored at −40 °C till used for assessment of ferritin, Transferrin (TF), soluble transferrin receptor (sTfR), EPO, sEpoR, ACE, and cytokines (IL-1β, IL-6, and IL-10) content, iron workup (iron and total iron-binding capacity; TIBC). Iron (µg/dL) and TIBC (µg/dL) were assayed using colorimetric kits (Stanbio Laboratory, Boerne, TX, USA). Transferrin saturation (%) was calculated from iron and TIBC. Plasma proteins and cytokines were assayed using specific quantitative commercially available ELISA kits as instructed; ferritin in ng/mL and sTfR in nmol/L (Diagnostic Automation/Cortez Diagnostics Inc, CA, USA; cat#1601-16 and 3126-15), TF in mg/dL (Abcam, Cambridge, MA, USA, USA cat#ab187391), ACE in ng/mL and sEpoR in ng/mL (MyBioSource, Inc., San Diego, CA, USA; cat#MBS494753 and MBS702997), IL-1β, IL-6, and IL-10 in pg/mL (RayBiotech, Inc., Peachtree Corners, GA, USA; cat# ELH-IL1b, ELH-IL6, and ELH-IL10), and Epo in mIU/mL (BioVision, Inc., Milpitas, CA, USA; cat# E4720-100). An aliquot of whole blood was also used to assess Hb, TLC count using a cell counter (Sysmex XT-4000*i* Automated Haematology Analyzer Lincolnshire, IL, USA). Hb level was measured in the 6th month three times, one week apart, the mean of these three readings was recorded. Half of the whole blood sample collected was used for genomic DNA extraction and real-time PCR analysis of ACE genes polymorphism.

Whole Blood Genomic DNA Extraction and ACE Genotyping and Allelic Discrimination

Total DNA was isolated from whole blood mononuclear cells (MNC) using the extraction kit (Zymo Research, Irvine, CA, USA; cat# D302 Quick-DNA Microprep Kit) instructed. The DNA purity (*A*260/*A*280 ratio) and concentration were assessed spectrophotometrically (dual-wavelength Beckman, Spectrophotometer, USA). GAPDH house-keeping gene was assessed in all PCR reactions as an internal control and for DNA integrity. The extracted and purified DNA samples were stored at −80 °C till used. ACE polymorphism genotyping and allelic discrimination was assessed using TaqMan Analysis. DNA was genotyped for ACE G/A at rs4343. PCRs were carried out in reaction volumes of 25 µL containing 50 ng DNA, 10 µL TaqMan Universal PCR Master Mix (Applied Biosystems, ThermoFisher Scientific Inc., Waltham, MA, USA) with the passive reference ROX (Perkin Elmer), 280 nmol/L of each primer and 200 nmol/L VIC-labeled probes for ACE G > A. Primers and minor groove binder probes were synthesized by Applied Biosystems. The primer sequence was

Table 1 General Characteristics and Laboratories of HD Patients versus Controls

	Groups	N	Value ± SEM	P value
Sex	Patients (Male) Patients (Female) Control (Male) Control (Female)	162 103 122 38		0.001
Age (year)	Patients Control	265 160	51.3 ± 11.9 36.1 ± 12.8	0.00
Potassium	Patients Control	265 160	3.79±.06 4.16±0.04	0.000
Urea	Patients Control	265 160	69.4±1.67 27.05±0.51	0.000
Creatinine	Patients Control	265 160	3.29±0.082 0.9±0.02	0.000
Serum Iron	Patients Control	265 160	43.24±0.64 131.28±2.52	0.000
TIBC	Patients Control	265 160	228.81±3.51 260.4±3.33	0.002
Transferrin Saturation	Patients Control	265 160	19.94±0.41 50.26±1.045	0.000
Ferritin	Patients Control	265 160	158.6±2.3 190.9±3.1	0.893
Transferrin	Patients Control	265 160	239.21±4.4 289.3±4.92	0.006
sTfR	Patients Control	265 160	17.86±0.4 11.28±0.09	0.000
Hb	Patients Control	265 160	10.69±0.09 13.8±0.07	0.000
HCT	Patients Control	265 160	33.21±0.73 33.24±0.7	0.015
WBC	Patients Control	265 160	4.39±0.08 6.86±0.17	0.000
Platelet	Patients Control	265 160	186.5±4.11 270.8±7.08	0.000
IL6	Patients Control	265 160	150.44±7.09 43.08±1.63	0.000
IL10	Patients Control	265 160	22.68±0.75 71.48±2.21	0.000
IL1b	Patients Control	265 160	43.29±1.46 8.95±0.78	0.000
Epo	Patients Control	265 160	15.44±0.34 68.8±5.11	0.000
ACE	Patients Control	265 160	54.35±1.3 270.3±8.09	0.000

(*Continued*)

Table 1 (Continued).

	Groups	N	Value ± SEM	P value
sEBoR	Patients Control	265 160	89.42±1.35 312.1±11.7	0.000

Note: Evaluated by independent *T*-test.
Abbreviations: ACE, Angiotensin-converting enzyme; TIBC, total iron binding capacity; Transferrin Sat, Transferrin Saturation; sTfR, soluble transferrin receptor; Hct, haematocrit; Epo, erythropoietin; sEpoR, soluble erythropoietin receptor.

forward: 5′-GTGAGCTAAGGGCTGGA-3′ and reverse: 5 ′-CCAGCCCTCCCATGCCCATAA-3′. PCR thermal cycler conditions included an initial incubation at 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 35 cycles of 15 seconds at 92 °C and 1 minute at 60–62 °C. Allele discrimination was accomplished by running endpoint detection using the StepOne and SDS 2.0 software. ACE AA = ACE Insertion/Insertion (I/I), ACE GA = ACE Insertion/Deletion (I/D) while ACE GG = ACE Deletion/ Deletion (D/D).

Statistical Analysis

Data were collected, tabulated, and analyzed using SPSS version 21 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). Deviation of genotype frequencies of the studied group of patients from Hardy-Weinberg equilibrium (HWE) was assessed by Chi-squared test with one degree of freedom (df) using the Michael H. Court's (2005–2008) calculator.[13] If $P \geq 0.05$, then the population is in HWE. For categorical data like gender was presented as frequency and percentage. Scale data like age, haematological parameters were presented as mean ± Standard Error of Mean (SEM). Shapiro–Wilk test was applied to determine the distribution of data. Chi-square test/ Fischer exact test was applied to measure the difference among categories. Independent samples *t*-test was used to measure the mean difference across two categories. Levene's test was applied to ascertain equal variance among the groups. One-way ANOVA with LSD posthoc analysis was applied to determine the difference in scale data among more than two categories. Correlations between ACE level and haematological parameters were using Pearson's correlation coefficient. The stepwise regression test was used to determine the independent parameters that may affect Hb or Hct values. A p-value < 0.05 was considered significant.

Ethical Approval

The current study protocol was approved by the Bioethics Committee, Medical College, Cairo University (Approval Number CU III F 40 20) and conducted following the Helsinki declaration.

Results

Changes in the Investigated Parameters in HD Patients vs Healthy Controls

Comparing HD patients vs healthy controls showed significant differences in plasma potassium, urea, creatinine, iron, TIBC, % TF Saturation, TF, sTfR, Hb, Hct, TLC, platelets count IL-6, IL-10 and IL-1β, EPO, ACE and sEpoR (Table 1).

Prevalence of ACE G2350A (Rs4343) Genotypes and Alleles Among HD Patients and Healthy Controls

The prevalence of ACE G2350A (rs4343) I/D genotype among *HD* patients and healthy controls showed that the I/D genotype is the most prevalent while the I/I genotype is the least one. ACE G2350A (rs4343) I/D genotype distribution showed a significant difference in the gene allele distribution between HD patients compared to normal controls: I/D (n = 174 vs 85), I/I (n = 41 vs 6) and D/D (n = 50 vs 69) (p = 0.001). D allele is the most prevalent one either in HD patients (0.52) or among the control group (0.7) (Table 2).

Relationship Between the ACE G2350A (Rs4343) Genotypes and Hb and Iron Levels in HD Patients

The mean Hb was highest in D/D genotype patients (11.12 ±0.19), followed by I/I (11.11±0.2) n I/D (10.47±0.1).

The effect of ACE G2350A (rs4343) genotypes on different parameters among CRF patients was evaluated using one-way ANOVA; a significant difference between the three categories was found, F= 5.9, P=0.003. Differences were significant between I/I and I/D genotype (mean difference=−.63, P = 0.012), D/D and I/D genotype (mean difference =−.65, P = 0.005). no significant difference was noted between I/I and D/D (P=0.956) Table 3.

Table 2 Patients and Control Group ACE Rs4343 Genotype and Allele Distributions

Group	ACE Gene*			ACE Allele**	
	I/I	I/D	D/D	I Allele	D Allele
Patient (Number) %	41 15.5%	174 65.7%	50 18.9%	0.48	0.52
Control (Number) %	6 3.8%	85 53.1%	69 43.1%	0.30	0.70

Notes: *P= 0.001. **P= 0.013. Evaluated by Chi-Square test.

Table 3 Comparison of Hb & Serum Iron in Different HD Patient Genotypes of ACE Gene Rs4343

	I/I	I/D	D/D
Hb (gm/dl) Serum Iron (μg/dl)	11.11±0.2 40.95±1.3	10.47±0.1* 44.5±0.9#	11.12±0.19 40.6±1.1

Notes: *Significance compared to II (P=0.012) and DD (P=0.005). #Significance compared to II (P=0.045) and DD (P=0.018). Evaluated by ANOVA.

The mean serum iron was highest in I/D genotype patients (44.53 ±.87), followed by I/I (40.95±1.3 n DD (40.6±1.05). A one-way ANOVA found a significant difference between three categories, F= 4.062, P=0.018. Differences were significant between I/D and II (mean difference=3.58. P =0.045), I/D and D/D (mean difference=3.93, P =0.018). I/I and D/D had not shown a significant difference (P= 0.871) Table 3.

Relation of the ACE G2350A (Rs4343) Genotypes and the Inflammatory Markers in HD Patients

There were insignificant differences among patients with I/I, D/D, or I/D genotypes regarding TLC (Figure 1A) or the inflammatory biomarkers (IL-6, IL-10, and IL-1β) (Figure 1B).

Relationship Between the ACE G2350A (Rs4343) Genotypes and Iron Status in HD Patients

There were insignificant differences among patients with I/I, D/D, or I/D genotypes regarding % TF Saturation and sTfR (Figure 2A), TIBC, Ferritin, or TF level (Figure 2B).

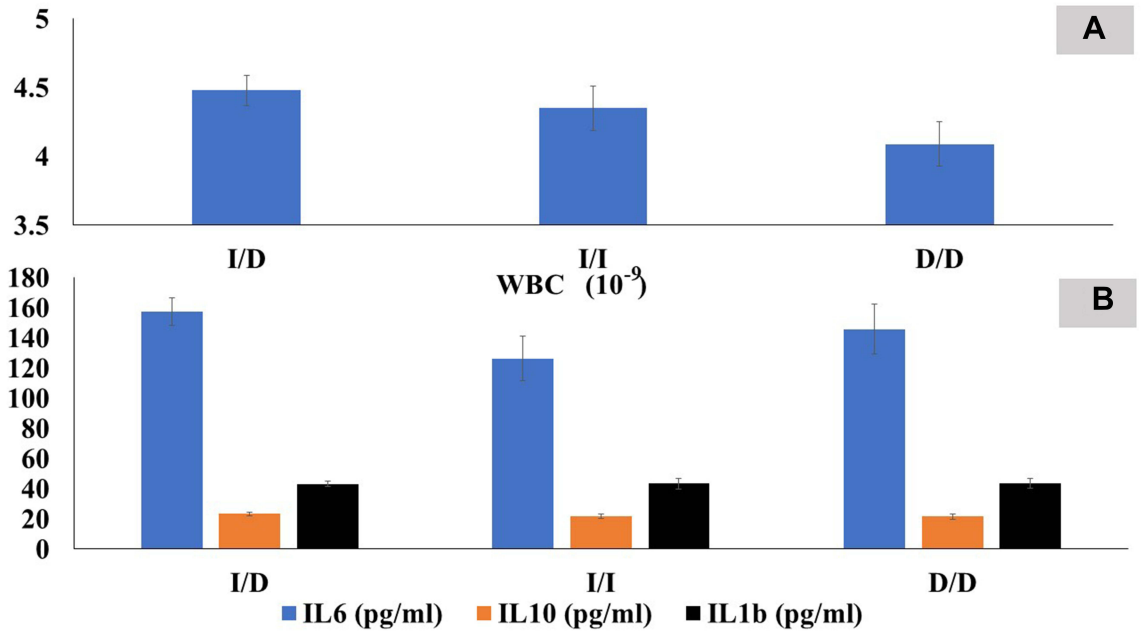


Figure 1 Comparison of WBC (**A**), IL6 & IL10 & IL1β (**B**) regarding the ACE G2350A (rs4343) genotypes. Data presented as mean ± SEM. Evaluated by ANOVA test followed by LSD as a post hoc.

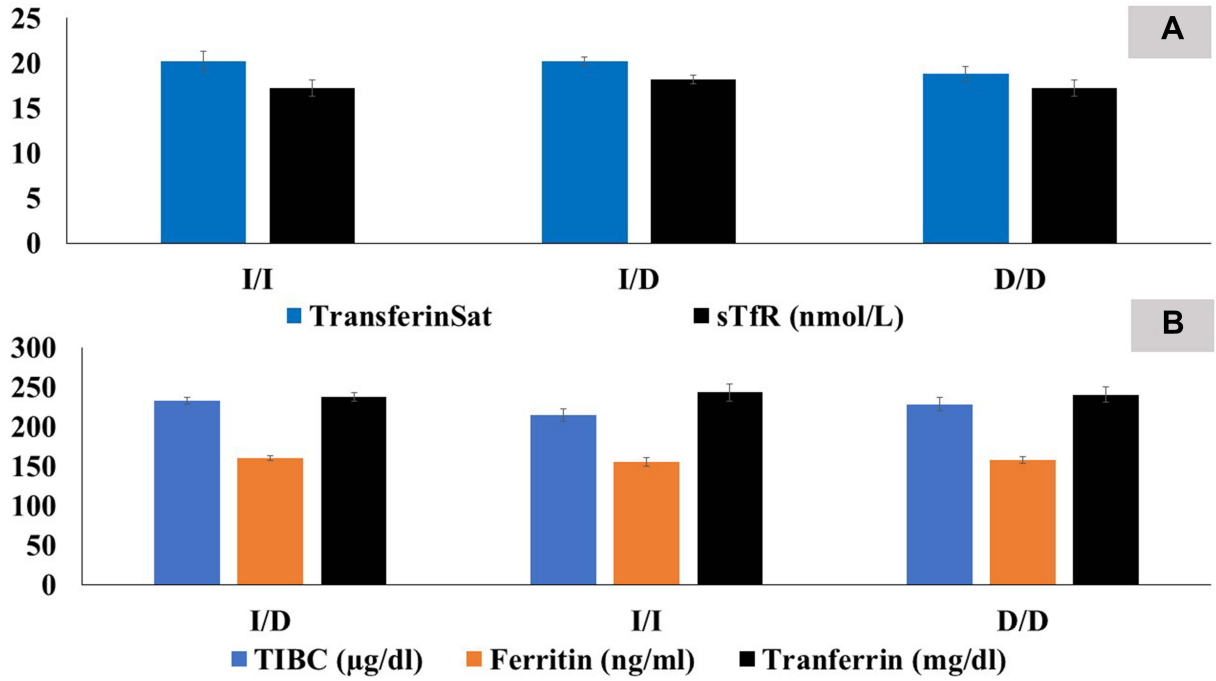


Figure 2 Comparison of Transferrin Saturation or sTfR (soluble transferrin receptor) (**A**), TIBC (Total Iron Binding Capacity), ferritin, and Transferrin (**B**) regarding the ACE G2350A (rs4343) genotypes. Data presented as mean ± SEM. Evaluated by ANOVA test followed by LSD as a post hoc.

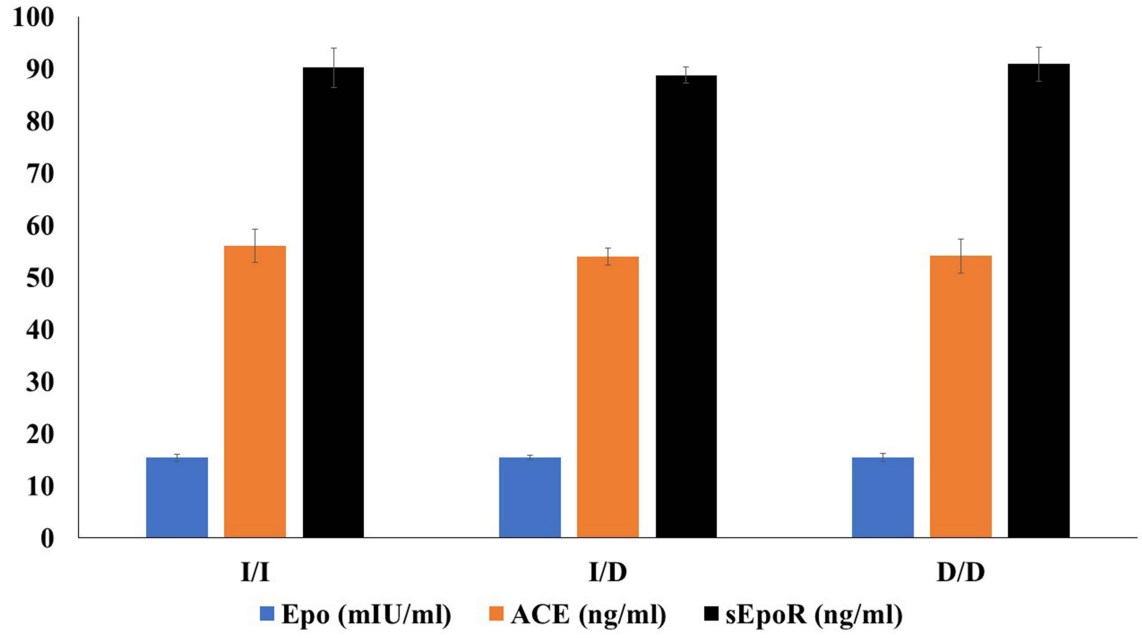


Figure 3 Comparison of Epo (erythropoietin), ACE (angiotensin-converting enzyme) and sEpoR (Soluble erythropoietin receptors) regarding the ACE G2350A (rs4343) genotypes. Data presented as mean ± SEM. Evaluated by ANOVA test followed by LSD as a post hoc.

Relationship Between the ACE G2350A (Rs4343) Genotypes and ACE, Epo, and sEpoR Levels

The effect of ACE G2350A (rs4343) genotypes on levels of ACE, EPO, and sEpoR levels was evaluated among CRF patients. Our results showed insignificant differences between patients with different genotypes in that regard (Figure 3).

Comparison Between the ACE G2350A (Rs4343) DD Genotype and Non-DD Genotype (ID±II) Patients

The D allele is the most prevalent allele among patients in the current study (Table 2). Analysis of the genotype correlation in a recessive mode of inheritance of the risk of D allele between Non-DD (II+ID) vs (DD) was done using an independent *t*-test. Our results showed a significant difference between the two groups regarding iron status (43.9±.7, 40.6±1.1, respectively, F: 6.946, t: 2.529, CI: 0.7019:5.8004, P=0.013) and Hb level (10.6±.1, 11.1 ±.19, respectively, F: 0.261, t: −2.308, CI: −0.9797:0.0776, P=0.013) (Table 4).

Correlations Between ACE Level and Haematological Parameters Among HD Patients

Using Pearson's correlation coefficient, the correlation between the ACE level and haematological parameters among HD patients showed a significant positive correlation between the ACE level and Epo (r: 0.244, P=0.0001) and a significant negative correlation between the ACE level and HCT (r: −0.131, P=0.033) (Table 5).

Linear Regression Test to Determine the Independent Factors Affecting Hb and Hct Levels Among HD Patients

Linear regression analysis revealed that among all parameters tested, ACE G2350A (rs4343) (R.194, P=0.001), TLC (R 0.282, P=0.001), and sEpoR (R 0.312, P=0.024) were independent predictors of Hb level (Table 6). While the ACE content (R. 0.292, P= 0.017), TLC (R. 0.255, P=0.015), and iron (R 0.209, P=0.001) were independent predictors of the Hct level (Table 7).

Discussion

The current study is the first report that studied the effect of ACE G2350A (rs4343) gene polymorphism on the haematological indicators of response to rHuEpo therapy. It is well-established that genetic factors play an essential role in determining the efficacy and response to drug treatment.[14] Pharmacogenomics analyses such relationships towards the personalization of medicine. Our lab showed the importance of such an approach in predicting the patient's response to different drug therapy.[15,16]

The present study showed that HD patients with the ACE G2350A (rs4343) D/D and I/I genotype respond better to rHuEpo therapy than those with the I/D genotype as evidenced by the higher Hb level among the former group. This higher Hb level among D/D and I/I genotypes were not related to iron level. Our results showed that patients with the I/D allele had higher iron than patients with each of the D/D and I/I genotypes, despite the lower Hb level of the I/D allele holders. The better Hb response was recently partially reasoned to higher plasma angiotensin II (Ang II) levels in D/D and I/D genotypes compared to the II genotype.[17]

Ang II is the main effector member of the renin-angiotensin system acting through the AT1 receptor and is generated from Ang. I by an ACE-induced proteolytic cleavage.[18] The Renin-angiotensin system plays a vital role in hematopoiesis and other diseases.[19,20] However, the exact mechanism by which ACE may affect erythropoiesis and Hb level is still not well elucidated. Among the other plausible explanations is ACE inhibition of Ang II–induced Epo release and prevention of the induction of pluripotent hematopoietic stem cells.[21] ACE directs stem cell differentiation to erythroid progenitors' synthesis.[22] ACE may affect the Ang II level, directly increasing erythroid progenitors' in vitro proliferation.[23]

Savin et al showed that the ACE D/D genotype is associated with higher Hb levels.[24] Patients with the D/D genotype were shown to require less Epo dose than the I/I genotype.[11]

In a study that included 112 ambulatory peritoneal dialysis patients, Sharples et al[25] showed that the ACE DD genotype requires less rHuEpo than other ACE genotypes, I/I or I/D. This result seems to be in line with our conclusion, albeit we could not identify the exact ACE SNPs that Sharples and his colleagues had examined. Similarly, Hatano et al[26] showed that HD patients with D/D-allele require low rHuEPO.

Table 4 Comparison of Different Parameters Between Non-DD (ID+II) and DD Genotype Among HD Patients

		Mean ± SEM	F	t	P value	95% Confidence Interval of the Difference	
						Lower	Upper
Serum Iron	Non-D (ID+II) DD	43.9±0.7 40.6±1.1	6.946	2.529	0.013	0.7019	5.8004
TIBC	Non-D (ID+II) DD	228.9±3.8 228.5±8.7	0.441	0.048	0.962	−17.264	18.121
Tranferritin Saturation	Non-D (ID+II) DD	20.2±0.48 18.8±0.78	2.267	1.359	0.175	−0.644	3.512
Ferritin	Non-D (ID+II) DD	158.9±2.7 157.4±4.3	1.674	0.252	0.801	−10.158	13.138
Transferrin	Non-D (ID+II) DD	238.9±4.9 240.5±9.6	1.875	−0.136	0.892	−23.887	20.799
sTfR	Non-D (ID+II) DD	17.9±0.45 17.3±0.89	0.255	0.713	0.476	−1.292	2.761
Hb	Non-D (ID+II) DD	10.6±0.1 11.1±0.19	0.261	−2.308	0.022	−0.9797	−0.0776
Hct	Non-D (ID+II) DD	33.3±0.84 32.7±1.4	0.574	0.367	0.714	−2.983	4.351
WBC	Non-D (ID+II) DD	4.5±0.09 4.1±0.16	1.847	1.734	0.084	−0.0493	0.776
IL6	Non-D (ID+II) DD	151.5±7.9 145.7±16.5	0.116	0.321	0.748	−29.941	41.605
IL10	Non-D (ID+II) DD	22.9±0.82 21.5±1.7	0.012	0.743	0.458	−2.337	5.1688
IL1b	Non-D (ID+II DD	43.2±1.6 43.8±3.3	0.322	−0.158	0.875	−7.945	6.767
Epo	Non-D (ID+II)) DD	15.4±.38 15.4±.82	0.689	0.006	0.995	−1.728	1.738
ACE	Non-D (ID+II) DD	54.4±1.4 54.1±3.3	1.360	0.092	0.927	−6.186	6.789
sEpoR	Non-D (ID+II) DD	89.1±1.5 90.9±3.3	0.566	−0.556	0.578	−8.756	4.898

Notes: Independent *t*-test. The number of non-DD genotypes (II+ID) is 215 while DD genotype patients is 50.

Abbreviations: ACE, Angiotensin converting enzyme; TIBC, total iron binding capacity; Transferrin Sat, Transferrin Saturation; sTfr, soluble transferrin receptor; HCT, haematocrit; Epo, erythropoietin; sEpoR, soluble erythropoietin receptor.

The ACE rs4646994 D/D genotype was associated with a poor response to rHuEpo in HD Korean patients, suggesting that it could be a useful genetic tool in predicting Epo requirement and responsiveness in HD patients.[10] Kiss et al,[27] working on Hungarian and Al-Radeef et al,[28] working on Iraqi HD patients, reported that ACE polymorphism had a non-significant effect on the Hb level. These variations may arise from the exact SNPs tested; we explored the ACE G2350A (rs4343) effect while they examined rs1799752 and rs4646994, respectively. Also, the small sample size of these studies compared to ours might have affected their conclusions.

Our results showed a higher iron store among the heterozygous ID genotype than II or DD genotype patients assuming a heterozygous advantage for the ACE G2350A

Table 5 Correlations Between ACE Level and Haematological Parameters Using Pearson's Correlation Coefficient

	ACE Level	
	r	P-Vlaue
Serum Iron	0.054	0.377
TIBC	−0.015	0.803
Transferrin Sat.	0.044	0.479
Ferritin	−0.001	0.982
Transferrin	−0.047	0.446
sTfR	0.108	0.081
Hb	0.074	0.233
Hct	−0.131*	0.033 *
Epo	0.244**	0.000 **
sEpoR	−0.044	0.479

Notes: *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).
Abbreviations: ACE, Angiotensin converting enzyme; TIBC, total iron binding capacity; Transferrin Sat, Transferrin Saturation; sTfR, soluble transferrin receptor; Hct, haematocrit; Epo, erythropoietin; sEpoR, soluble erythropoietin receptor.

(rs4343) ID genotype among HD patients included in the present study.

Heterozygote advantage or overdominant refers to better fitness of heterozygous genotype patients over both homozygous. It firstly appeared in 1922 to maintain polymorphism stability.[29] Major histocompatibility complex (MHC) gene represent one of the prominent examples for the heterozygote advantage, in which MHC heterozygotes genetic diversity is abundant. Heterozygote genotype patients have better recognition of pathogen antigen and resist infections effectively than homozygous.[30,31] Heterozygote advantage provides a protective effect against malaria for the sickle-cell anaemia allele carriers.[32]

Recently, A genome-wide association study revealed that heterozygous individuals were significantly healthy-aged compared to other individuals with other genotypes. Moreover, in the same age group population, a 10-year higher survival was associated with individuals with higher heterozygosity rates; the association is more likely to be explained by heterozygote advantage.[33] Previous observations noted heterozygous advantages on ACE genotype patients among cardiovascular diseases; because of high linkage disequilibrium (LD) between the polymorphisms, *ACE* haplotypes needed to be determined in different populations with different evolutionary histories search for additional ancestral breakpoints. The phenotypes' complexity also includes the possibility of multiple interactions between genes or genes and environmental factors. The high frequency of I/D, ie, 56.61%, could be because of heterozygote advantages against the two homozygotes D/D and I/I in cardiovascular diseases[9] and kidney diseases; individuals with I/D genotype have the least levels of ACE. The DD genotype has the highest levels, followed by I/I[34] or having lower plasma ACE levels,[35] although these studies may differ from our study in its design, ethnicity, and allele distributions.

A 287-bp insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene (17q22-q24, 26 exons, and 25 introns) in humans may control serum ACE levels. Many SNPs in linkage disequilibrium (LD) with the I/D polymorphism, including T5941C, A262T, T93C, T1237C,

Table 6 Hb Stepwise Regression Test

	R	R2	Adjusted R2	Coefficient	SE	Standardized Coefficient		P value
ACE gene	0.194	0.038	0.034	0.360	0.112	0.194	3.210	0.001
WBC	0.282	0.079	0.072	0.225	0.065	0.205	3.441	0.001
sEpoR	0.312	0.097	0.087	0.009	0.004	0.134	2.275	0.024

Note: Dependent variable: Hb.
Abbreviations: R, correlation coefficient; SE, standard error; P, significance probability; WBC, white blood cell count; ACE gene, Angiotensin converting enzyme gene; sEpoR, Soluble Erythropoietin receptor.

Table 7 HCT Stepwise Regression Test

	R	R2	Adjusted R2	Coefficient	SE	Standardized Coefficient	P value
Serum Iron	0.209	0.043	0.040	0.238	0.069	0.209	0.001
WBC	0.255	0.065	0.058	1.294	0.528	0.146	0.015
ACE	0.292	0.085	0.075	−0.080	0.034	−0.142	0.017

Note: Dependent variable: HCT.
Abbreviations: R, correlation coefficient; SE, standard error mean; P, significance probability; WBC, white blood cell count; ACE, Angiotensin converting enzyme.

C4656T, and A11860G (*rs* 4343; exon 16),[36,37] are known to influence serum ACE.[38]

Furthermore, rs1799752 is one of four SNPs that may be the most well-studied ACE SNP. It is an insertion/deletion of an Alu repetitive element in an ACE gene's intron rather than a single nucleotide polymorphism.

ACE G2350A (rs4343) gene polymorphism is associated with increased ACE enzyme activity in physiological and pathological states.[39] It increases ACE levels in subjects with a high-saturated-fat diet that enhances diet-dependent hypertension.[40]

Our data showed insignificant differences among the tested three ACE G2350A (rs4343) I/I, I/D, and D/D genotypes regarding the circulating ACE protein content. On the contrary, Mizuiri et al and Elshamaa et al demonstrated an opposite conclusion. ACE I/D genotype is associated with renal ACE gene expression in healthy Japanese subjects[41] and plasma and tissue ACE levels.[42] Nand et al showed D allele positively affects ACE serum level.[43]

Endogenous or rHuEpo binds to EPOr resulting in stimulation of erythropoiesis.[44] sEpoR is generated from mRNA alternative splicing, and since it lacks the transmembrane domain, it is released into extracellular fluids. sEpoR buffers rHuEpo because of its high affinity to EPO; therefore, it acts as a potent antagonist to EPO, resulting in decreased response to rHuEpo treatment. sEpoR high level was correlated to a high need for rHuEpo dose.[45,46]

In the current work, there was an insignificant difference between ACE G2350A (rs4343) I/I, I/D, or D/D genotypes regarding plasma Epo and sEpoR content in the present study. This notion contradicts the finding of Al-Radeef et al, who showed that another rs1799752 I/D and D/D genotypes had a higher serum Epo level compared to the I/I genotype.[28]

Our patients were free of active infection, and the measured proinflammatory cytokine levels, IL-6, IL-1β, and IL-10, were insignificant differences among the three ACE G2350A (rs4343) genotypes; I/I, I/D, or DD.

Increases in the inflammatory mediator, such as IL-6 and TNF-α, lead to increases in the sEpoR level that would hinder erythropoiesis.[46] sEpoR stabilizes proinflammatory cytokine ligand and modulates cytokine interaction to its membrane-bound receptor, leading to variation in its concentration.[47] Inflammatory cytokines accompanying CRF and HD decrease rHuEpo efficacy. TNF-α, IL-1, and IL-6 induce resistance against rHuEpo in erythroid progenitor cells reducing iron release from the reticuloendothelial system and decreasing Hb production.[48,49] Betjes et al reported a lack of response to rHuEpo among CKD patients with cytomegalovirus infection mainly due to IFN-γ and TNF-α production.[50]

Although our HD patients showed higher levels of % TF saturation and sTfR, TIBC, Ferritin, or TF, there were insignificant differences among patients with I/I, D/D, and I/D genotypes regarding these parameters.

Various tissues obtain their iron need via TF binding to its receptor, endocytosis of the complex, and iron download.[51,52] The expression rate of the cell surface TF receptor is directly proportional to its iron need.[53] The transmembrane glycoprotein TF receptor is formed of two disulfide-linked monomers; each polypeptide subunit comprises three major domains: a large C-terminal extracellular domain and a transmembrane and an N-terminal cytoplasmic domain. sTfR is the cleaved extracellular domain of the high-affinity iron-sensor TF receptor released soluble in extracellular fluids. Circulating levels of sTfR reflect the number of cells with receptors (erythropoietic activity) and the receptor density on cells (tissue iron status).[54] Ferritin is used for diagnosing iron deficiency anaemia, but it could be falsely elevated in inflammation giving the erroneous impression of normal iron stores.[55] sTfR is insensitive to inflammatory states and inflammatory biomarkers. It could detect anaemia even in subjects with the inflammatory condition; moreover, it could differentiate between anaemia due to iron deficiency or chronic diseases.[56]

Finally, we tested for independent factors that may affect the patient's response to rHuEPO. Among all parameters tested, ACE protein level, TLC, and sEpoR were the independent predictors of Hb level. Simultaneously, ACE protein content, TLC, and iron are the independent predictors for the Hct level.

Limitation of the Current Study

Previous works measured Hb level at the beginning, 3rd, and 6th months of treatment with rHuEpo [24, 28]. In the present study, we measured the Hb level after six months of the treatment with rHuEpo to allow more precision and avoid fluctuation of patient response to treatment. We took the mean of the three Hb levels in the 6th month. We could not retrieve accurate data considering the use of ACE inhibitors (ACEIs) or ARBs among our patients. We measured circulating ACE level as a protein rather than an activity that revealed insignificant differences among the three genotypes assessed to avoid any related confusion. We did not evaluate angiotensin II (Ang II) level in the

current study and iron intake status, but we estimate Hct, iron, ferritin, TF, % TF saturation, sTfR, and TIBC. Many other ACE gene SNPs may affect the HD patient's response to rHuEPOs as rs1799752, rs429, and rs4341 which may be in linkage disequilibrium with studied rs4343; however, the only studied here is the ACE G2350A (rs4343). These limitations of the current study are highly acknowledged and will be considered in our future studies.

Conclusion

Patients with either ACE G2350A (rs4343) I/I or D/D genotype showed better response to rHuEpo than those with I/D genotype. ACE protein content, TLC, and sEpoR may represent independent predictors for the HD patients' response to rHuEPOs. Screening for ACE G2350A (rs4343) gene polymorphisms in the HD patients on HD before rHuEpo administration may predict patients' response.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors stated that they have no conflicts of interest for this work.

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