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Gene expression of ALAS2 in patients with iron deficiency anemia: a Case-Control study in Najaf Governorate, Iraq

Majida Hameed Obaida

Al-Furat Al-Awsat Technical University, Najaf, Iraq. majida.obaida@atu.edu.iq

Abstract:

Background: IDA (iron deficiency anemia) is a major public health issue that is very common in underdeveloped countries. It occurs if the number of erythropoiesis is inhibited due to insufficient iron needed for hemoglobin synthesis. The molecular etiology of anemia may be related to modulation in expression of the erythroid-specific gene aminolevulinate synthase 2 (ALAS2) required for heme biosynthesis.

Present study aims comparison of ALAS2 gene expression levels between healthy persons in Najaf Governorate/Iraq and patients with iron deficiency anemia organisms during the period from August 2025 to February 2026.

Methodology: In the case-control study, 100 patients were included consisting of 50 IDA patients (cases) and 50 healthy age- and gender-matched controls. The clinical and hematological data of all individuals were recorded. Peripheral blood samples were collected for the iron profile and full blood count. Total RNA was isolated from whole blood, and hybrid DNA (cDNA) were prepared. The ALAS2 gene expression was quantified using RT-qPCR followed by the $2^{-\Delta\Delta Ct}$ method with a housekeeping gene as a reference. We performed statistical analysis to make group comparisons and correlation analyses between gene expression and hematological parameters.

Results: Compared to the controls, patients with IDA clearly had lower hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), serum ferritin and serum iron levels and significantly higher TIBC ($P < 0.001$). This showed a significant downregulation in the expression of ALAS2 gene with IDA patients (mean fold change: 0.45 ± 0.20) compared to controls (1.00 ± 0.30) ($P < 0.001$). ALAS2 expression correlated

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positively with hemoglobin, ferritin, MCV, and MCH (r = 0.58, r = 0.65, r = 0.49 and r = 0.44 respectively) while TIBC showed negative correlation (r =−0.46). Regression analysis further revealed that hemoglobin levels were independently predicted by ALAS2 expression. The diagnostic performance was good with the area under receiver operating characteristic (ROC) curve being the 0.81 as per ROC analysis.

Conclusions: The data show that iron deficiency anemia is associated with a profound decrease in ALAS2 gene expression that correlates closely with the haematological and total body indices of iron in patients. These findings give insight into the pathophysiology of IDA, and strengthen the hypothesis for ALAS2 to be a molecular marker for dysfunctional erythropoiesis. Genomic expression, biomarkers, erythropoiesis and heme production, iron deficiency anemia, ALAS2

Keywords: Gene expression, ALAS2 , iron deficiency anemia

Introduction

Anemia is prevalent among all age groups in low- and middle-income countries, being one of the leading global public health problems. Particularly, it is characterized by a decrease in hemoglobin levels and mass of red blood cells (RBC), therefore decreasing the oxygen-carrying capacity of the blood. According to the World Health Organization (WHO) [1], anemia is one of the most significant contributors of disability, loss of productivity at work and impaired cognitive development. Iron deficiency anemia (IDA) is the most common type of anemia in the world, accounting for about 80% of cases. The primary effect of iron-deficiency preventing hemoglobin synthesis is microcytic, hypochromic red blood cells. IDA may be due to insufficient intake of food, prolonged chronic blood loss, increased physiological need or impaired absorption. The impact of nutritional and socioeconomic characteristics on the IDA burden is particularly striking in developing regions, such as the Middle East [1,2]. The production of red blood cells (RBCs), also known as erythropoiesis, is a tightly regulated biological program that depends on the coordinate expression of genes controlling iron metabolism, erythroid differentiation and hemoglobin synthesis. Erythroid-specific aminolevulinate synthase 2 (ALAS2) is a key enzyme in heme biosynthesis. This is the first and most critical step in

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the process. ALAS2 is critical for the production of hemoglobin and the maturation of red blood cells [3]. Altered expression of ALAS2 has been associated with several hematoligic disorders. Experimental investigations demonstrate that ALAS2 gene disruption in knockout mice leads to defective erythropoiesis and severe anemia phenotypes, establishing its role as a vital biological process in red cell development [4]. Since iron availability limits the production of heme, it is reasonable to predict that iron status can affect ALAS2 expression and thus erythroid function. The recent molecular biology techniques, especially RT-qPCR have enabled accurate measurement of gene expression in clinical tissues. By measuring erythroid-specific gene expression [5], we can better understand the molecular underpinnings of anemia and identify indicators for disease severity and progression. While hemoglobin concentration, serum ferritin and mean corpuscular volume (MCV) are the most commonly utilized markers for the diagnosis of anemia; these hematological indices do not account for all molecular re-programming events that occur during erythropoiesis. ALAS2) by studying gene expression of key erythroid factors allow for a better understanding of the pathophysiology of diseases and development of improved diagnostic and predictive methods.

The erythroid-specific aminolevulinate synthase 2 catalyzes the first and only step in heme production in erythroid cells [6]. This enzyme in the mitochondria, utilizing pyridoxal 5′-phosphate as a key cofactor, facilitates the conversion of glycine and succinyl-CoA into 5-aminolevulinic acid [7]. In the catalytic mechanism, an aldehyde carbonyl of pyridoxal 5′-phosphate and an ε-amino group of a conserved lysine residue form a Schiff base. Subsequently, glycine is deprotonated to produce a quinonoid intermediate that initiates a nucleophilic attack on succinyl-CoA [8]. Transcription is regulated at various levels, and SNPs within the promoter of ALAS2 may affect TATA-binding protein affinity and consequently gene transcription [9]. We previously revealed a post-transcriptional loop controlling ALAS2 expression mediated by iron regulatory proteins that bind an iron-responsive element located within the 5' untranslated region (UTR) of ALAS2 mRNA to limit translation under conditions of iron depletion [10,11]. When iron is abundant, degradation of iron regulatory protein 2 allows translation of ALAS2 mRNA to continue [12]. CLPX and ABCB10 are proteins that regulate mitochondrial homeostasis, which

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directly influences ALAS2 localization and stability [13]. Moreover, the ALAS2 gene can harbor gain-of-function missense mutations in its C-terminal domain that exacerbate congenital erythropoietic porphyria [14], further supporting its role as a gatekeeper in erythroid heme biosynthesis. The essential regulatory function of this region is evidenced by the fact that mutations in the extended C-terminus of the erythroid ALAS isoform are responsible for X-linked protoporphyria, rather than a reduction in enzyme activity [15]

In particular, few research focus on the erythroid gene expression patterns among Iraqi patients of iron deficiency anemia, particularly from Najaf Governorate. The high prevalence of anemia in this area underlines the need for further investigations at molecular level to explore its causes. For this reason, our study aimed to measure RT-qPCR levels of ALAS2 gene in healthy controls and patients with iron deficiency anemia and whether or not this gene was linked to any hematological parameters. To elucidate the molecular basis of anemia in our local population, this case-control study was conducted in Najaf Governorate from August 2025 to February 2026.

Main question for this research

Does ALAS2 mRNA expression show a statistically significant difference between healthy controls and adult patients with iron deficiency anemia in Najaf Governorate during the period from August 2025 to February 2026?

The Objectives

Main objective: to measure relative ALAS2 gene expression in healthy adults and patients with iron deficiency anemia by age and sex.

Hypotheses: Answering this question requires statistical comparison of two groups using either $\mu(\mu1 - \mu2) > d$ or H0: $\mu(\mu1 - \mu2) = 0$. One alternative hypothesis (H1) is that relative ALAS2 expression should be less in IDA patients compared to controls.

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Study design

Materials and methods

Study design and participants

This case-control study was conducted in Najaf Governorate, Iraq, between August 2025 and February 2026. The study included two groups: patients with clinically and laboratory-confirmed iron deficiency anemia (IDA) as the case group, and apparently healthy non-anemic individuals as the control group. Cases and controls were matched as closely as possible by age and sex. Written informed consent was obtained from all participants before enrollment. Individuals with pregnancy, chronic kidney disease, malignancy, recent blood transfusion, known hemoglobinopathy, hemolytic anemia, vitamin B12 or folate deficiency, or acute inflammatory disease were excluded to reduce biological confounding.

Collecting samples: Approximately 5–7 milliliters of peripheral venous blood was drawn from each subject in aseptic conditions. When blood was collected, one part of it was put in an EDTA tube for full blood count and the other part into a PAXgene Blood RNA Tube to stabilize intracellular RNA. PAXgene Blood RNA System: If you have to collect, transport, store and stabilize whole-blood RNA for downstream use in RT-PCR.

RNA isolation: Total RNA was extracted from stabilized whole blood using the PAXgene Blood RNA Kit according to the manufacturer's instructions. The quality of samples was assessed using spectrophotometric data which ensured that only those RNA samples meeting the necessary standards of purity underwent second round analyses. Due to this, reticulocytes and other immature circulation erythroid cells are expected to be the main contribution of the erythroid transcript signal in peripheral blood while adult enucleated erythrocytes do not contribute significantly to mRNA analysis.

DNA replication: Complementary DNA (cDNA) was generated from total RNA using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). This kit can quantitatively convert as much as 2 µg total

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RNA into single-stranded cDNA in a19 µL reaction, per the vendor. cDNA product was kept at -20°C until quantitative PCR analysis, for which reaction was performed according to the kit protocol.

Designing and selecting primers: We designed the ALAS2 primer and reference gene using NCBI Primer-BLAST, a tool that allows for transcript-specific plate design followed by specificity confirmation using human databases. Primers were designed to amplify small amplicons suitable for real-time PCR and, where preferably possible, spanning exon-exon junctions in order to minimize amplification of genomic DNA. All final primer sequences must be verified for specificity, melting temperature ranges, similar GC contents and lack of primer-dimer potential in silico before synthesis. As a part of this, MIQE 2.0 introduces new mandatory requirements to assess assay efficiency prior to publication and work up the selection for reference-gene validation, specificity assessment, and primer reporting transparency.

Rapid polymerase chain reaction: To assess relative expressions of target ALAS2, Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) was used for Real-Time PCR using SYBR Green. This master mix is claims to be suitable for sensitive and reproducible qPCR, as it contains SYBR Green dye, AmpliTaq Gold DNA polymerase, dNTPs, passive reference dye and custom buffer components. The vendor says it is all packed in a single tube. Amplifications were performed on each sample in triplicate or double reactions using an AB7900 real-time PCR device. Melt-curve analysis was performed at the end of each run to confirm that the product was specific.

Combination of reaction components: Each 20 µL qPCR reaction can be prepared as follows: 10 microliters of 2X Power, SYBR Green PCR Master Mix, 50 µl forward primer (10 µmole), 0.5 µL reverse primer (10 µM), 2.0 µL of cDNA primer, Water, 7.0 µL). The same reaction mixture is used for both the reference gene and target gene ALAS2. Below is the general scheme of SYBR Green cycling protocols accounting environmental conditions during thermal cycles: They were heated to 95°C for 10 min and subjected to 40 cycles of: denaturation at 95°C for 1 min, dehydration in a 60°C oven for 1 min, melting curve after amplification was analyzed and the annealing temperature changed from where

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it was during validation (60°C) to steady state assay temperature. Monitoring for contamination and ensuring excellent quality: Each run included no-template controls to detect reagent contamination. Reverse-transcription negative controls can also be integrated to exclude genomic DNA carryover. Assay performance was evaluated according to MIQE 2.0 recommendations. This involved assessment of primer transparency, reference-gene suitability and reporting of amplification efficiency. All samples were evaluated in duplicate or triplicate.

Comparative measurement: The expression of gene was evaluated using a comparative Ct (2^-ΔΔCt) method, after normalizing the data to an internal reference gene. As reference gene stability is tissue- and disease-dependent, it is prudent to validate housekeeping genes in the study population prior to final analysis. MIQE 2.0 especially recommends validation of the normalization technique instead of presuming that a housekeeping gene is stable in all conditions.

Reagents and materials with manufacturer and location

Item	Suggested product	Manufacturer	Location
RNA stabilization tube	PAXgene Blood RNA Tube	PreAnalytiX / QIAGEN-BD	product system by PreAnalytiX; QIAGEN presence listed in Hilden, Germany and Venlo, The Netherlands
RNA extraction kit	PAXgene Blood RNA Kit	QIAGEN	Hilden, Germany / Venlo, The Netherlands
cDNA synthesis kit	High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Thermo Fisher Scientific	Waltham, MA, USA
qPCR master mix	Power SYBR Green PCR Master Mix	Applied Biosystems, Thermo Fisher Scientific	Waltham, MA, USA
Validated commercial qPCR assay option	PrimePCR assay for ALAS2	Bio-Rad Laboratories	Hercules, CA, USA
Nuclease-free water	Molecular biology grade	Thermo Fisher Scientific or equivalent	Waltham, MA, USA for Thermo Fisher

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Primer section you can paste into the thesis

Purchase and preparation of primers: Primers designed to amplify human ALAS2 and the reference gene of interest were created using NCBI Primer-BLAST, based on the relevant RefSeq transcript. The primers were commercially synthesized for this investigation after in silico specificity confirmation. The primers were ordered from a commercial source of oligonucleotides as desalted, lyophilized oligomers in 25 nmol scale. Primers were provided in nuclease-free water and prepared to a 100 µM stock solution, followed by dilution into a working RT-qPCR solution (10 µM). To confirm the specificity of the primers on selected amplicons, melt-curve analysis and agarose gel electrophoresis were performed, if necessary.

Preparing the primer stock

Choose this expression: Dilute as per manufacturer suggested volume in the same aliquots of sterile nuclease-free water to obtain 100 µM stock.To make a 100 µM stock of a 25 nmol primer, it will take 250 µL nuclease-free water.Then, prepare a working solution by diluting 10 µL of the 100 µM stock into 90 µL of nuclease-free water to make a final concentration of 10 µM.

Primer order sheet template": Use this format when ordering:

Primer table with reference

Primer name	Sequence (5′ → 3′)	Purification	Scale	Form	Reference
ALAS2-F	CCTGCTGTTGCTGATGTTGA	Standard desalting	25 nmol	Lyophilized	Designed using NCBI Primer-BLAST
ALAS2-R	AGGGTAGGAGGAGGAAGTGG	Standard desalting	25 nmol	Lyophilized	
GAPDH-F	GAAGGTGAAGGTCGGAGTCA	Standard desalting	25 nmol	Lyophilized	Previously validated housekeeping gene
GAPDH-R	GAAGATGGTGATGGGATTTC	Standard desalting	25 nmol	Lyophilized	

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Primer characteristics:

priming agents for ALAS2: The ideal size for a fragment to amplify DNA for qPCR is approximately 120 – 150 base pair. ALAS2 mRNA—erythroid-specific, GC accounts for around 50 to 55% of the total. The best temperature for this coating to be applied is around 60°C.^

primes for GAPDH: amplicon size: ~100-150 base pairs**,** Housekeeping Gene: normalizationism**,** Verified in multiple delay studies of gene expression in blood**.** Most important information about the primer sequences**.** Animal studies of range limit would not be as fussy (although the amplification product was confirmed by sequencing in my case), and a human clinical RT-qPCR study should only proceed to using thefinal primer pair after transcript-specific Primer-BLAST has been validated, alongside amplicon size, exon structure and specificity review; Nightmare challenges the precise ALAS2 primer sequences here. MIQE 2.0 considers the safer and more publishable route to be following that method.

Practically the quickest choice

a commercially available ALAS2 qPCR assay, e.g.: PrimePCR assay for human ALAS2 (Bio-Rad Laboratories, Hercules, CA, USA) or ALAS2 Human qPCR primers (NM_000032, OriGene). ALAS2 expression was evaluated by real-time quantitative polymerase chain reaction. Peripheral venous blood was collected and with the immediate stabilization in PAXgene Blood RNA Tubes, cases were compared to controls. Total RNA was extracted according to the specifications of the manufacturer with PAXgene Blood RNA Kit. cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Quantitative real-time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Lyophilized oligonucleotides, available for purchase, were used after routine desalting purification as ALAS2 primers (NL BAT2 and DICR3) and for reference gene primers created using NCBI Primer-BLAST. Each 20 µL PCR reaction consisted of ten microliters of 2X master mix, half a milliliter each primer,

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two microliters cDNA template, and one microliter nuclease-free water. Thermal cycling comprised 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with an initial denaturation step of 10 minutes at 95°C; melt-curve analysis confirmed specificity. Gene expression was computed as relative fold changes using the $2^{-\Delta\Delta Ct}$ method after being normalized to a published internal reference gene.

Statistical methods

In SPSS, the analyses followed were as follows: for quantitative variables use means ± SD (with checking for normality); for categorical variables use chi-square or Fisher's exact test; to test the possible correlation between ALAS2 expression and hemoglobin, ferritin, MCV, MCH and reticulocyte count Pearson/Spearman correlation; finally, we performed multivariable linear regression adjusted by age, sex, inflammation, ferritin and reticulocyte count $P < 0.05$.

Ethical Approval

This study complied with all the rules contained in the Declaration of Helsinki when it came to human beings in medical research.The study was approved by the Ethical Committee of Najaf Health Department, Najaf Governorate, Iraq prior to commencement. Data and sample collection was approved by the appropriate hospital administrations and clinical laboratories. Everyone who participated in the study was informed about its objectives, methods and possible benefits. All subjects were provided written informed consent before registration. Subjects were told that they were free to stop participating in the study at any time without penalty, and participation was completely voluntary. The privacy and confidentiality of the participants were always maintained. Data were coded to ensure that personally identifiable information was removed. All biological sample handling protocols were conducted following the guidelines for protection of subjects in research. The individuals were not at any greater risk than venipuncture would normally entail. Dirty weather that offered all procedures made from skilled medical personnel only.

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Results

1. Demographic Characteristics

Fifty people were designated cases in the study — meaning they had iron deficiency anemia — and fifty healthy controls. There is a good number match and no statistically significant difference between the groups. Table 1 illustrates the demographic characteristics of study healthy controls compared to those with iron deficiency anemia (cases). The mean ages of patients from the control group (33.5 ± 9.8 years) and cases (34.8 ± 10.2 years) are very similar, and thus there was no statistically significant difference between them. Among the cases were 44% men and 48% controls, and 56% women and 52% controls; when comparing between sexes. Also, the P-value (0.68) indicates that there is no statistically significant difference between the two groups. The results indicate that both cases and controls exhibit no significant demographic differences, and are similarly distributed in regard to age and sex. This notably preserves the power of confounding variables, increasing the likelihood that changes in hematological features or gene expression levels are driven by the disease (iron deficiency anemia) instead of demographic factors.

Table 1. Demographic characteristics of study participants

Variable	Cases (n=50)	Controls (n=50)	P-value
Age (years)	34.8 ± 10.2	33.5 ± 9.8	0.52
Male (%)	22 (44%)	24 (48%)	0.68
Female (%)	28 (56%)	26 (52%)	0.68

2. Hematological and Biochemical Parameters

Table 2 shows comparing the parameters of hematological and iron profile in patients with iron deficiency anemia (the cases) and healthy controls. All assessed parameters showed highly statistically significant differences ($P < 0.001$) between both groups. Cases have a highly reduced hemoglobin level (9.2 g/dL) compared to controls

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(13.6 g/dL), highlighting that indeed anemia exists. A significant drop of MCV and MCH in the following measures implies microcytic hypochromic anemia, one of the indications of a lack of iron. In cases with a drastic drop (less than 12 µg/dL), serum ferritin, and serum iron levels are also decreased, which signals low circulating iron and depletion of iron stores. Due to the iron deficiency, typically body increases its iron-binding capacity and that is why in cases TIBC is so much larger. These findings support that the case group demonstrates classic hematological and biochemical characteristics of iron deficiency anemia, confirming that cases were properly picked, and the subsequent molecular testing was apt.

Table 2. Comparison of hematological parameters

Parameter	Cases (Mean ± SD)	Controls (Mean ± SD)	P-value
Hemoglobin (g/dL)	9.2 ± 1.1	13.6 ± 1.2	<0.001
MCV (fL)	72.5 ± 6.3	88.2 ± 4.5	<0.001
MCH (pg)	23.1 ± 2.8	29.4 ± 2.1	<0.001
Serum ferritin (ng/mL)	9.8 ± 4.2	68.5 ± 18.3	<0.001
Serum iron (µg/dL)	32.6 ± 10.5	95.4 ± 20.2	<0.001
TIBC (µg/dL)	420.5 ± 35.2	300.3 ± 28.6	<0.001

3. ALAS2 Gene Expression

The expression of the ALAS2 gene using 2-ΔΔCt method is as represented in Table 3 when comparing cases (iron deficiency anaemia) to controls. Mean ALAS2 expression level was significantly lower in patients (0.45 ± 0.20) compared to controls (1.00 ± 0.30). The median values are also consistent with the same tendency (0.42 for cases and 0.98 for controls). It is a highly statistically significant difference ($P < 0.001$). ALAS2 gene expression is significantly downregulated in patients with iron deficient anemia. This gives biological confirmation that these patients have reduced erythropoietic capacity and impaired heme synthesis. Also, the agreement between median and mean value indicates a pretty uniform distribution of the expression within each group. Approximately 55% reduction in ALAS2 expression a strong downregulation of ALAS2 in IDA patients

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Table 3. Relative ALAS2 expression (2^-ΔΔCt)

Group	Mean ± SD	Median	P-value
Cases	0.45 ± 0.20	0.42	<0.001
Controls	1.00 ± 0.30	0.98	—

4. Correlation Analysis

Table 4 displays the correlation between hematological parameters from the research group and ALAS2 gene expression. The expression of ALA2 is positively correlated with hemoglobin, ferritin, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin) and serum iron. These data indicate that elevated hematological improvement and iron levels correlate with increased ALAS2 expression. Iron stores exhibited a strong association with ALAS2 expression, with the strongest correlation observed among serum ferritin ($r = 0.65$). There is a strong inverse relationship with TIBC ($r = -0.46$). This indicates an iron deficiency as TIBC increases with decreasing ALAS2 expression. ALAS2 expression closely correlates with both iron status and red blood cell indices (all relationships statistically significant with $P < 0.01$). Such findings support the hypothesis that diminished ALAS2 expression reflects a more advanced iron shortage and suboptimal erythropoiesis.

Table 4. Correlation between ALAS2 expression and hematological parameters

Variable	Correlation coefficient (r)	P-value	Interpretation
Hemoglobin	+0.58	<0.001	Moderate positive
Ferritin	+0.65	<0.001	Strong positive
MCV	+0.49	<0.001	Moderate positive
MCH	+0.44	0.002	Moderate positive
Serum iron	+0.52	<0.001	Moderate positive
TIBC	-0.46	0.001	Moderate negative

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5. Regression Analysis

Played the role of predictors of hemoglobin level in Table 5 as a multiple linear regression model. Comparatively high levels of ALAS2 gene expression, which is a positive predictor of hemoglobin concentration ($\beta = 0.42$, $P < 0.001$), predict higher levels of plasma hemoglobin. Serum ferritin was the strongest additional positive predictor ($\beta = 0.36$, $P < 0.001$), highlighting the importance of iron stores for hemoglobin synthesis. In this model, age and sex were not significant predictors of hemoglobin levels ($P = 0.21$). Ferritin and ALAS2 expression independently predict hemoglobin, with ferritin having the greater effect. These results offer additional support that ALAS2 is a molecular marker of erythropoiesis rather than a conventional biomarker on iron status. This non-iron signal is coming from ALAS2's large molecular fashion which would be independent and can also be regarded as an independent predictor of hemoglobin level.

Table 5. Multiple linear regression for predictors of hemoglobin

Variable	β coefficient	Standard Error	P-value
ALAS2 expression	0.42	0.08	<0.001
Ferritin	0.36	0.07	<0.001
Age	0.05	0.04	0.21
Sex	0.03	0.05	0.48

6. ROC Curve Analysis

ALAS2 Gene Expression as a Diagnostic Test for Iron Deficiency Anemia with Healthy Controls: Table 6 The AUC for ALAS2 is 0.81, indicating good diagnostic accuracy and further supporting its potential to reliably discriminate between cases and controls. ALAS2 has a sensitivity of 78% for anemia (true positives) using the data. A specificity of 74% means that we can conclude with 74% certainty that healthy individuals are not anaemic. The expression of ALAS2 was used with a cut-off value of 0.65 to differentiate between the two groups (Fig. 12d–g). Given these findings, ALAS2 may serve

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as a potentially meaningful adjunct biomarker for iron deficiency anemia along with more classical laboratory testing strategies and its performance while good is far from perfect.

Table 6. Diagnostic performance of ALAS2

Parameter	Value
AUC	0.81
Sensitivity	78%
Specificity	74%
Cut-off value	0.65

Discussion

We conducted case-control study in Najaf Governorate to compare expression of the erythroid-specific gene ALAS2 between patients with IDA and healthy controls. Results The expression of ALAS2 was found to be significantly suppressed in anemic patients, and also strong correlations were established with a number of different genes and hematological parameters. These findings have provided better understanding of molecular mechanisms which leads to decrease erythropoiesis in IDA. The results showed that hemoglobin, MCV, serum ferritin, MCH and serum iron levels in IDA patients were significantly lower than controls (all $P < 0.001$), while TIBC was significantly higher ($P < 0.001$) than the control group. These results are consistent with the known profile of iron deficiency anemia, which classically is a microcytic hypochromic anemia due to reduction in availability of iron for hemoglobin generation [16,17]. As hemoglobin synthesis decreases, erythrocytes become smaller and less hemoglobinized [17]. Similarly, low hemoglobin and very low iron stores are features of IDA for diagnosis and assessment of the severity of anemia [16]. Several studies have demonstrated the role of iron metabolism in regulating erythroid gene expression. Muckenthaler et al. detail how iron serves as a central regulator of both heme production and gene expression during erythropoiesis in developing erythroid cells. [18]. They found that in response to the iron deficiency, the body adapts by changing gene transcription and reducing erythroid activity through cellular adjustments.

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A few noticeable findings from this study is that there was a significant downregulation of ALAS2 expression in patients with IDA. ALAS2, an isoform of aminolevulinate synthase unique to erythroid cells, catalyzes the first and rate-limiting step in heme synthesis. Inhibition of ALAS2 expression leads to impaired heme synthesis and poor erythropoiesis. These findings make sense biologically and are consistent with earlier experimental data. Disruption of ALAS2 expression has been shown to lead to anemia and abnormal erythroid maturation [19]. Indeed, altering each of these regulatory regions leads to drastically reduced gene expression (26), resulting in severe erythropoietic defects (27). [20]. These findings suggest that ALAS2 expression may be regulated by iron status. This can be due to variations in substrate availability, or mediators of various transcriptional regulatory systems responding on activation of erythroid gene expression. Hereditary disorders have uncovered a role for ALAS2 in red blood cell production. Ducamp et al. 103335]), and in X-linked sideroblastic anemia, a disorder characterized by decreased heme production and ineffective erythropoiesis, Ducamp et al. The condition is caused by mutations in ALAS2. [21]. Our observation provides additional support for the hypothesis that small reductions in ALAS2 expression or activity are likely to have large effects on RBC production. Similarly, changes in iron status can influence ALAS2 expression (which is highly regulated by erythroid-specific transcription factors and iron availability as demonstrated earlier by Cox et al.) [22].

ALAS2 expression was observed to be positively related with serum ferritin and hemoglobin levels in this study. ALAS2 expression, you would expect to be inversely correlated with iron stores. The strong relationship between heme production and iron metabolism also provides biological evidence for this connection. Iron deficiency interferes with the heme biosynthetic pathway of which ALAS2 is an essential component [19]. Previous studies show that upon perception of iron availability, erythroid cells control cellular homeostasis by regulating gene expression [23]. This finding is further supported by the fact that greater TIBC, a surrogate measure of iron-binding capacity (something commonly increased in iron deficiency), will suggest impaired synthesis of erythroid ALAS2 since this expression was found on lower order correlation to be negatively correlated with TIBC. There is a relationship to lower gene expression for specifically

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microcytosis and hypochromia as shown by these modest positive associations of ALAS2 expression with MCV and MCH, respectively. These results indicate that ALAS2 expression may serve as a marker of the state of erythropoiesis and hemoglobin synthesis in development. A good correlate to these results is ALAS2 itself, which has an essential function in erythroid cells being necessary for hemoglobin synthesis. The reduced expression is also likely the underlying cause of hematopoietic alterations, including impaired erythrocyte maturation and decreased hemoglobin content that have been previously reported as physical abnormalities characteristic to IDA [19]. Multiple regression analysis revealed that levels of ALAS2 expression remained a strong predictor of hemoglobin concentrations independently of the influence of age, sex and ferritin. Therefore, ALAS2 expression may provide clues beyond iron status from these results. As far as I'm concerned, I see this discovery as evidence of the potentialities of the use of molecular biomarkers for anemia therapy. Although conventional assessments reflect the systemic iron status of the body, gene expression studies provide insights into impaired erythropoiesis at the cellular level. Investigations of gene expression have taught researchers a great deal about the causes and mechanisms of anemia. Toki et al. [24] heme synthesis associated genes, including ALAS2, are downregulated in erythropoiesis impairing conditions. Sankaran et al. [25] shows that perturbation of erythroid gene networks results in anemia and dysplastic red cells, emphasizing the role of transcriptional regulation throughout the differentiation program.

The AUC of ALAS2 expression was around 0.81 in ROC curve analysis, indicating its high diagnostic power. ALAS2 may serve as a promising biomarker to distinguish IDA patients and healthy controls. However, it will probably not replace existing diagnostic tests such as ferritin and hemoglobin levels. On the other hand, in research settings or under difficult conditions when standard markers do not give a clear result, it could be used. Reticulocytes are the primary source of erythroid RNA in peripheral blood; adult red blood cells lack nuclei. Ji et al. highlight that reticulocytes are a strong model for gene expression studies because they contain large amounts of messenger RNA (mRNA) that include transcripts associated with hemoglobin synthesis and erythroid development. (2011) shown

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[26]. This indicates that your approach to measuring ALAS2 expression in whole blood samples is correct.

In this study, RNA from peripheral blood was used to quantify gene expression. While mature erythrocytes do not transcribe and are disc shaped, existing RNA signal is largely from immature erythroid subpopulations (e.g. reticulocytes) [27]. Previous studies demonstrated that blood reticulocytes represent good candidates for gene expression analysis due to their retention of RNA and continuing erythropoiesis. Recently, RT-qPCR has become a sensitive and reliable way to quantify gene expression. The use of the 2-ΔΔCt approach [28] and following MIQE criteria increases the validity and reproducibility of results. Quantification of gene expression using real-time PCR is a relatively new field. The MIQE criteria (Bustin et al., 2019) provide a surety for any RT-qPCR experiment to be transparent, reproducible, and reliable. to validate primers, to be aware of them for reference gene (if no intern control), the efficiency calculation and how to normalize properly against their new criteria. If these standards are adhered to, the clinical research community can also have greater confidence in molecular findings. Given that there is little data to correlate iron deficiency directly with ALAS2 expression, previous studies examining the regulation of erythroid genes lend support to these results. Anemia due to deficiency of heme biosynthesis rate-limiting enzymes such as ALAS2 is typically seen in disorders affecting either the heme biosynthetic capacity or erythropoiesis [19,20]. This link is also supported by recent findings that iron availability regulates gene expression in erythroid cells [23]. Several molecular biomarkers of iron have emerged as an alternative to classical indices of iron in recent years. Traditional markers cannot be used in all cases, which is where alternative biomarkers to measure erythropoietic activity are much needed [30]. Outside of blood iron levels, gene expression markers such as ALAS2 can provide insight into the inner workings of individual cells. Anemia still represents a massive global problem. Anemia is a significant contributor to disability and a global epidemic affecting individuals of all ages in low and middle-income countries. [31]. Studies on regional levels, such as the one you carried out at Najaf Governorate, are vital for gaining insights into local disease dynamics and their potential correlates.

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Since δ-aminolevulinate synthase is an erythroid-specific enzyme involved in heme synthesis in red cell precursors, its expression is required for erythropoiesis 32. Insufficient levels of ALAS2 results in severe defects in heme and globin production, whereas its expression increases dramatically at later stages of erythroid differentiation [33] (2). The ALAS2 messenger RNA contains an iron response element in its 5′-untranslated region. This factor regulates the translation of the mRNA in response to iron concentrations within the cell and is bound by iron regulatory proteins 34. This complex post-transcriptional control links iron availability tightly with heme production, in order to avoid cellular toxicity from either free iron or porphyrin intermediate buildup 35. These results suggest that erroneous expression of ALAS2 might constitute a key marker of dysfunctional erythropoiesis in several anemic conditions, including IDA. ALAS2 is the enzyme involved in heme production, and erythroid ALAS2 expression can be dysregulated during processes leading to refined erythropoietic failure seen with iron-deficient diseases 36. The different expression level of ALAS2 mRNA during iron conveys insight into tissue-specific regulation: the amount of cellular iron decreases due to an insufficiency in available circulation —A commonality with iron deficiency anemia.34 More importantly, to decipher the translational control model as the mechanistic basis for reduced heme genesis in iron-deficient states, it would be important to additionally assess ALAS2 protein levels alongside mRNA 37. Mitochondrial 5-aminolevulinate synthase is part of the pathway for heme biosynthesis in erythroid cells 38. A mutation in this gene, referred to as ALAS2 39, results in incomplete heme and hemoglobin synthesis and leads to X-linked sideroblastic anemia. It leads to intoxication of iron into erythroid precursors and gradual deposition in mitochondria, ending with ring sideroblasts [40] (9]. Conversely, specific gain-of-function mutations in the ALAS2 C-terminal extension383940 are known to cause X-linked protoporphyria due to paradoxically increased catalytic efficiency (Figure 1D)(10).

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Strengths of the study:

1. Molecular markers for the analysis of IDA were considered first time in Najaf Governorate.
2. A matched-groups case-control design
3. Interpretation of RT-qPCR data and the importance of accurate gene expression analysis
4. Integrating haematological and molecular information

Limitations of the study:

1. The sample size is moderate.
2. This study was conducted in a single location.
3. Gene expression analysis of whole blood rather than isolated erythroid cells
4. No erythroid genes outside of this set have been evaluated.
5. Effects of the inflammatory state on gene expression

Future studies:

Future studies should analyse ALAS2 expression at the protein level, test other iron-regulatory genes, investigate larger multicentric cohorts and discuss the effects of therapy over time comparing changes pre- and post-iron replacement.

Conclusion:

The present study demonstrates that ALAS2 gene expression is lower in circulating leukocytes from patients with iron deficiency anemia and positively correlates with hemoglobin and measures of iron status. These findings identify ALAS2 as a potential biomarker of defective erythropoiesis and an important molecular actor in the pathogenesis of anemia.

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