Role of the NR1H4 Genetic Variations and IgG Levels in Immune Thrombocytopenia Development in Children

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Abstract

Background: The idiopathic thrombocytopenic purpura also known as immune thrombocytopenic purpura (ITP) In children is one of the autoimmune disease classes of unknown etiology, The evaluation of Human Platelet Factor-4 (PF-4) levels in pediatric patients with ITP reveals significant differences when compared to healthy controls and links with NR1H4 gene expression profiles in pediatric patients with ITP provides critical insights into the molecular mechanisms underlying this condition, Spotlight the significant up regulation of NR1H4 with Immunologically and potential utility as a diagnostic biomarker.

Aim: the evaluated gene expression NR1H4 with immunological as a biomarker thrombocytopenia purpura development in children

Results: The comparison of NR1H4 gene expression and PF-4 between ITP patients and healthy control subjects has been carried out. Show result The Mean of NR1H4 gene expression were 8.34 ± 1.47, while the human pf-4 1.00 ± 0.44 and 11.99 ± 2.98 and 2.48 ± 0.79, in ITP patients and healthy control respectively. Wherefore, the mean levels in both gene expression and PF-4 were higher in ITP pediatric patients compared to healthy control and the difference was significant (P= 0.001)

Conclusion: The study demonstrated the gene expression of NR1H4 and the pathways by which PF-4 affects immune and platelet functions in ITP. The function of this gene may use a potential biomarker for the diagnosis of ITP. This could pave the way for novel therapeutic approaches ultimately improving management strategies for affected.

Keywords: Thrombocytopenic purpura, Human Platelet Factor-4, Pediatric patients, Iraq

Introduction

The thrombocytopenic purpura is an immune-mediated acquired disease of adults and children characterized by the transient or persistent decrease of the platelet count and, depending upon the degree of thrombocytopenia, increased risk of bleeding platelet count <100 × 10⁹/L (Provan *et al*., 2019; Abdaljabbar *et al*,. 2020) about children, ITP mostly shows acutely After contracting a viral infection, with most cases automatically resolved. However, 20-30% develops chronic ITP, requiring a deeper understanding of its cause (González *et al.*, 2023). Recent studies have focused on factors interacting between genetic predisposition and immune dysregulation, especially about immunoglobulin (IgG) autoantibodies. Genetic factors contribute largely to autoimmune disorders, Hanif et al 2021. In the case of ITP, IgG autoantibodies target platelet glycoproteins (e.g., GPIIb/IIIa), leading to platelet recognition for phagocytosis by splenic macrophages (Sinha *et al.*, 2023). High serum IgG levels, especially platelet-reactive IgG, are associated with disease severity and persistence in children (Cooper *et al.*, 2004; Saleem *et al.*, 2022). While, transient

IgG appearing may resolve acute ITP, persistent autoantibody production suggests an underlying immune defect, perhaps influenced by genetic variants (Al-Bayati *et al.*, 2021). Considered NR1H4 gene, for encoding the Farnesoid X receptor (FXR) is important because receives accountability due to the role of the dual combination in bile acid metabolism and immune regulation Kubelková. 2010 the FXR is expressed on immune cells, including B-lymphocytes with macrophages; however, receptors modulate inflammatory responses and maintain the tolerance of the immune system. The interaction between NR1H4 SNPs and IgG levels in ITP is still not fully explored. Functionally, when activated FXR suppresses NF-κB signaling, a pathway that is important for the proliferation of B cells and the production of antibodies Lu *et al.*, 2018. FXR dysfunction means that NR1H4 gene variants can enhance NF-κB activity, leading to excessive IgG synthesis. A recent study by Li et al., 2021 detected higher anti-platelet for IgG titers in children patients by ITP carrying the NR1H4 rs35724 G allele, Which indicates a hereditary immune reaction. Moreover, murine models show that FXR agonists decreased autoimmune responses, suggesting therapeutic potential Zhang *et al*. (2024). Therefore, research is focused on exploring the potential role of NR1H4 genetic variations and IgG levels in the development of pediatric thrombocytopenia.

Material and method

Samples

Collection 100 samples of blood after sterilizing the puncture area with 70% alcohol using disposable syringes. Blood samples were collected by drawing 5 ml sterile syringes from each examined. Fifty Patients with thrombocytopenia attending the Hemato Oncology Center and Al-Karama Teaching Hospital, and fifty controls, from a period between September 2023 and March 2024 and the ages of patients ranged (1-15) years

Measurement of Human Platelet Factor 4 (PF4)

ELISA kit was used in this study for quantitative determination of Human Platelet Factor 4 (PF4) from patient and healthy control serum samples according to the method described by Vayne *et al.* (2017), and was done according to the company's instructions, BT-LAB / Germany. While, in genetic screening using the quantitative Real-Time PCR was used in quantifying the NR1H4 gene expression analysis, which was normalized by the housekeeping gene (GAPDH). This method was carried out according to the method described by Xu *et al*. (2024), using a kit the

Total RNA Extraction Kit AccuZol™ from a company Bioneer /Korea. The result was calculated the expression analysis (fold change) was analyzed by using the Livak method, as described by Livak and Schmittgen (2001).

Statistical analysis

Data were collected, summarized, analyzed and presented using the statistical package for social sciences (SPSS) version 26 and Microsoft Office Excel 2010. In a manner, an independent sample t-test, One-way ANOVA test with Chi-square test was used to study the association between any two categorical variables. Pearson correlation was used to evaluate the correlation between any 2 numeric variables and the results were expressed as correlation coefficient (r) and the level of significance (P). The level of significance was considered at a P-value of less than 0.05 and highly significant level at 0.01 or less (Daniel and Cross, 2018; Jasim *et al*., 2024).

Result and Discussion

The present study enrolled 50 patients with ITP and 50 healthy control subjects. In the study evaluation of PF-4 levels in pediatric patients with ITP reveals significant differences compared to healthy controls, indicating its potential as a diagnostic marker. Specifically, are shown in (Table 1) and (Figure 1), PF-4 levels were markedly elevated in ITP patients (11.99 ± 2.98) versus controls (2.48 ± 0.79), with a highly significant p-value of 0.001, the result proven by the researcher Faraj and Ghali (2019) corroborating previous findings that suggest PF-4 is involved in the pathophysiology of ITP (Cuker *et al*., 2023; Mussbacher, *et al.*, 2024). The strong correlation between elevated PF-4 levels and ITP suggests a role for this cytokine in the immune dysregulation observed in these patients (Rashid *et al.,* 2020; Goel *et al.,* 2021).

Table 1: Human Platelet Factor-4 (PF-4) level in patients and healthy control

Groups		**(PF-4) level**
ITP pediatric patients	Mean ± SD	11.99 ± 2.98
	Range	4.72-21.90
Healthy control	Mean ± SD	2.48 ± 0.79
	Range	0.01-5.52
p-value		0.001** †

SD: standard deviation †: Independent T test; **: significant at $P < 0.05$

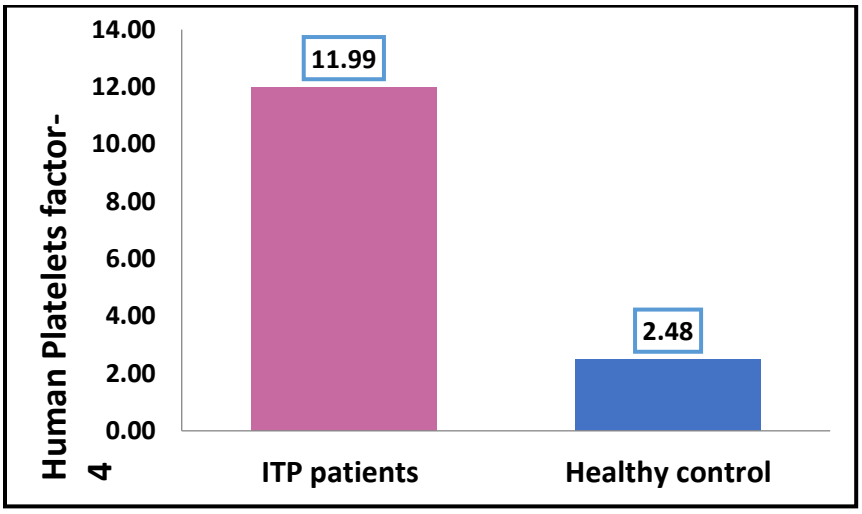

Figure 1: The means level of PF-4 in ITP patients and control groups

While the results showed the diagnostic accuracy of PF-4 was further validated by Receiver Operating Characteristic (ROC) Receiver operating characteristic analysis was performed to reveal the diagnostic accuracy of using PF-4 concentrations on ITP pediatric patients from healthy control subjects and the results are shown in (Table 2) and (Figure 2). The PF-4 cutoff value was > 5.30-fold with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), the result agrees with Tolba *et al*. (2023) while, the area under curves of 98.0%, 98.0%, 98.0%, 98.0% and 0.995 (0.987- 1.000). Indicating PF-4 could serve as an excellent biomarker for differentiating ITP and healthy controls group (Cines *et al.,* 2004; Neunert *et al.,* 2019). This aligns with existing literature that emphasizes the utility of PF-4 in various hematological contexts, including its role in platelet activation and immune response (Cabrera *et al.*, 2021; Bacsa *et al*., 2024). The present results indicate that PF-4 is considered an excellent diagnostic marker as mention by Audia *et al.* (2021).

Table 2: Sensitivity and specificity of PF-4 level (> 5.30-fold) in ITP

PF-4 level	**patients** ***n*** **= 50**	**Healthy control** ***n*** **= 50**
> 5.30	49 (%)	1 (%)
< 5.30	1 (%)	49 (%)
Sensitivity %	98.0 %	

Specificity %	98.0 %
PPV %	98.0 %
NPV %	98.0%
AUC (95% CI)	0.995 (0.987- 1.000)

CI: Confidence interval, AUC: Area under curve.

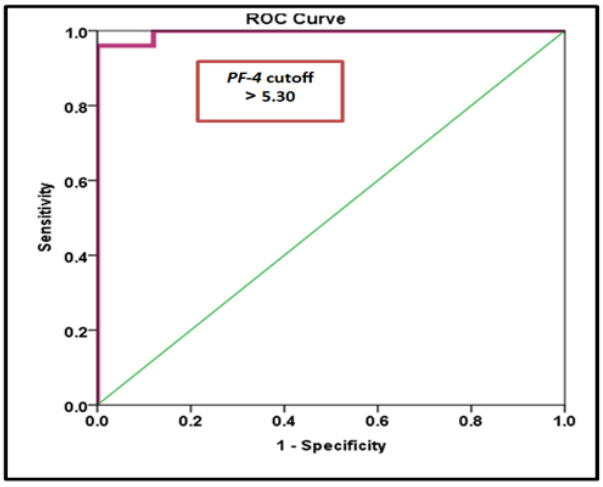

Figure 2: Receiver operator characteristic curve analysis of PF-4 for the calculation of possible diagnostic cutoff value

The findings underscore the complexity of ITP pathology, wherein factors like PF-4 may contribute to the disease's clinical manifestations without direct correlation to traditional hematological indices (Lambert *et al*., 2017; Audia *et al.*, 2021). Future research should focus on elucidating the specific pathways through which PF-4 influences immune and platelet function in ITP potentially offering new therapeutic targets (Provan and Semple, 2022; Cai et al., 2022). Given the critical role of PF-4 in immune response and platelet regulation, it may also serve as a valuable prognostic factor, guiding treatment decisions and improving patient outcomes (Lambert and Gernsheimer, 2017; Chen 2023.), Overall, the significant elevation of PF-4 in ITP patients presents an opportunity for further exploration into its role in the disease process and its application in clinical practice.

The exploration of gene expression patterns in pediatric patients with ITP provides critical insights into the molecular mechanisms that highlight this condition, highlighting the significant upregulation of NR1H4 and its potential utility as a diagnostic biomarker

Regarding genetic diagnosis, initially, the results showed that the purity of total RNA samples ranged from 1.65 to 1.92 ng/µl in the study groups with a mean ± SD of 1.84±0.12 ng/µl. The RNA purity of the study groups, as well as good yield with a high concentration of total RNA, depends on the extraction condition, (Liu *et al*., 2012). A high concentration of RNA was associated with no DNA. The main examination was carried out by Real-time PCR quantification, applied in the present experiment, which utilized the SYBR green, according to Schmittgen, and Livak, (2008).

Before Estimated Real time PCR Quantification of NR1H4 Expression detection, the Basic Assumption of the Household Molecular Genes Used Studies show that their expression remains constant in the cells or tissues underneath, according to Piergiorge *et al.*,(2024). One of the most common household genes associated with gene expression data is GAPDH (Kreplak, *et al*.,2019; Sellamuthu *et al*., 2022).

The Estimated Real-time PCR Quantification of GAPDH Expression by The Ct value of GAPDH, the housekeeping gene used in the present study, is the mean of the Ct value for GAPDH of the ITP patients' group, which was (23.13). In the control group, which was (23.54) as shown in (Table 3), the GAPDH amplification diagrams and dissociation curves Plots of each run were recorded, including the amplification plots and dissociation curves. Figures (3) show the amplification plots and dissociation curves for NR1H4, which indicates that the NR1H4 gene level did not seem to change in such compartments at ITP, as mentioned to the researchers, Alaa, R.(2024)

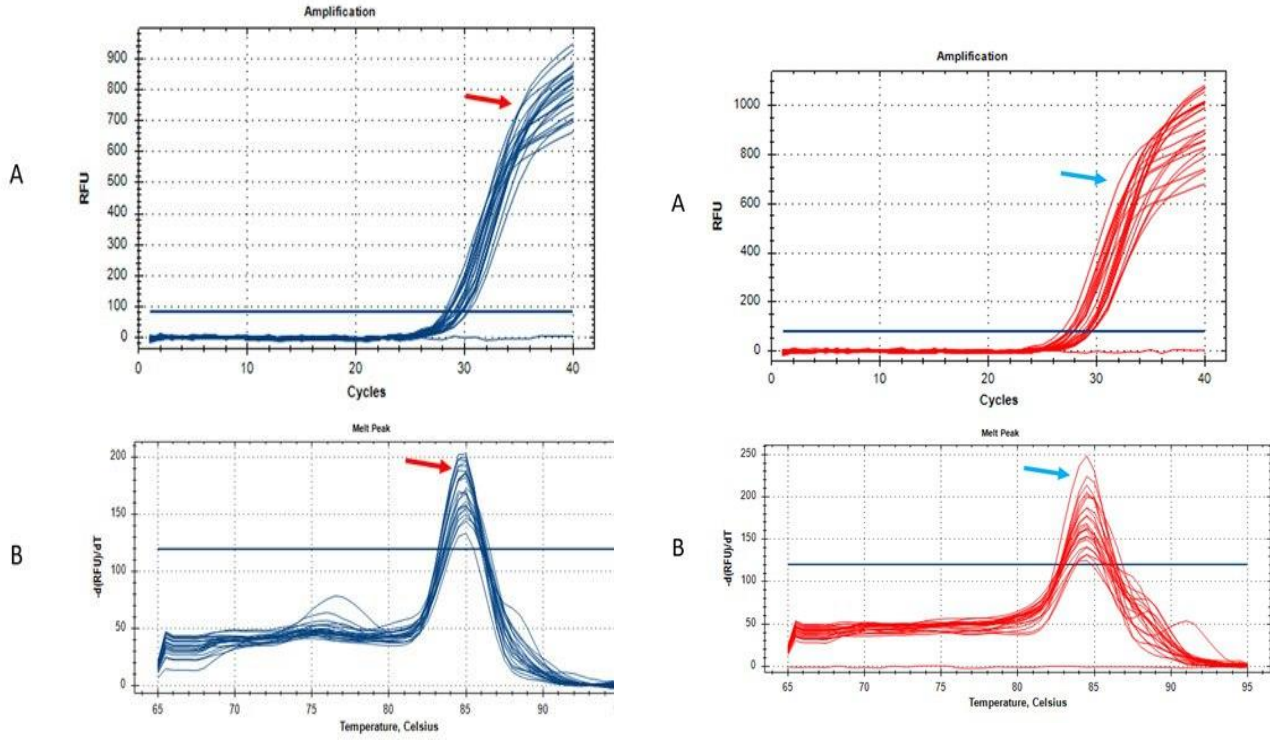

Figure 3: GAPDH and NR1H4 gene amplification plots by qPCR. Samples included all study groups. The photograph was taken directly from Agilent qPCR machine

Table 3: Comparison of (Ct, $^{2^{-\Delta}}$ct and Folding) between patients and healthy controls

Groups	Means Ct of NR1H4	Means Ct of GAPDH	$^{\Delta}$Ct (Means Ct of NR1H4)	$2^{-\Delta Ct}$	Fold of gene expression
ITP patients	25.96	23.13	2.83	-3.01	8.34
Control	29.38	23.54	5.84	-0.003	1.007

In the calculation of the relative expression of the NR1H4 gene in all study groups, the 2-ΔCt results were applied. A calibrator was used and it was one of the samples of the controls with high expression of NR1H4. As shown in (Table 3) the mean of 2-ΔCt values of the control group was (- 1.65) and that for ITP patients was (-3.01). When calculating, the gene expression was significantly higher in the ITP patients' group than in the control group.

The fold number in the ITP group was 8.34, as shown in (Table 4) and (Figure4). The results align with previous research indicating that NR1H4, as approved by Mjali, *et al*,. 2023, also known as liver X receptor alpha (LXRα), plays a pivotal role in regulating immune responses and

inflammation (Noelia and Castrillo 2011; Oger *et al.*, 2014) also This agrees with the researcher Xu et al, (2024).

The comparison of NR1H4 gene expression between ITP patients and healthy control subjects has been carried out, and the results are demonstrated in (Table 4) and (Figure 4). The mean of NR1H4 gene expression was 8.34 ± 1.47, and 1.00 ± 0.44 in ITP patients and healthy control respectively; the mean levels were higher in ITP patients compared to healthy control and the difference was highly significant (P < 0.001).

Table (4): Comparison of mean of NR1H4 gene expression between patients and healthy controls

Groups	Mean	SD	SE	p-value
ITP patients	8.34	1.47	0.68	0.001**
Control	1.00	0.44	0.25	

SD: standard deviation; SE: standard error;

†: one way ANOVA; **: significant at *P* < 0.05

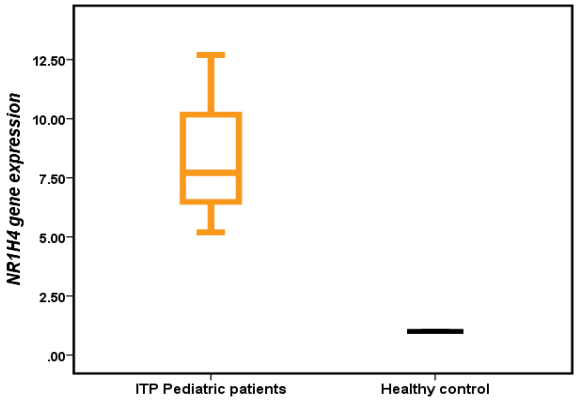

Figure 4: The means *NR1H4 gene expression* in patients and control groups

The significant difference in NR1H4 expression between the two groups (p < 0.001) further supports the hypothesis that alterations in lipid metabolism and immune regulation may contribute to the pathogenesis of ITP (Wang and Tontonoz, 2018; Nakashima *et al.*, 2024). While the results showed in Receiver operating characteristic (ROC) analysis were performed to reveal the diagnostic accuracy of using the NR1H4 gene to distinguish ITP patients from healthy controls. As shown in the (Table5) and (Figure 5) it turns out results indicate that the NR1H4 gene is considered an excellent diagnostic marker to distinguish ITP patients from healthy controls. (Cuker and Neunert, 2016; Ahmad *et al.*, 2023)

This is particularly significant given the challenges in diagnosing ITP, where clinical presentations can vary widely and overlap with other hematological disorders (Nagrebetsky *et al*., 2019 Kuter 2021; Saleem *et al.*, 2021). The utilization of quantitative reverse transcriptase real-time PCR (qRT-PCR) for assessing gene expression has been validated in various studies, proving to be a reliable method for quantifying mRNA levels (Schmittgen, and Livak, 2008; Shati, *et al* ., 2022.)

Table 5: Roc curve of *NR1H4 gene expression*

Characteristic	*ITP patients / control*
Cutoff value	> 3.09
P value	0.001
Sensitivity %	100.0 %
Specificity %	100.0 %
PPV %	100.0 %
NPV %	100.0 %
AUC (95% CI)	1.000 (1.000- 1.000)

CI: Confidence interval, AUC: Area under curve.

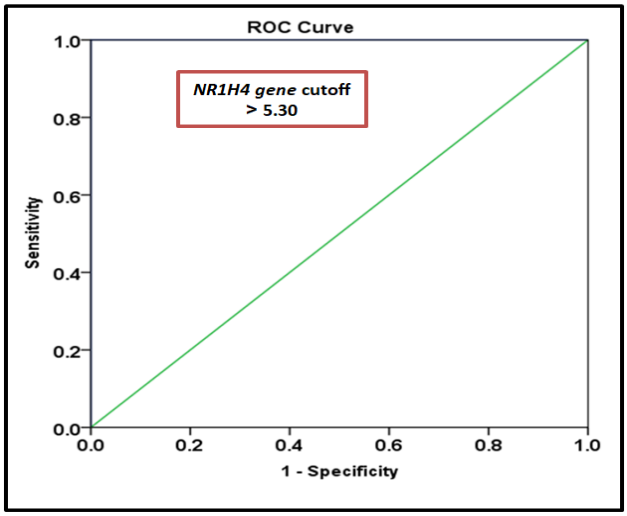

Figure 5 Receiver operating characteristic curve for NR1H4 gene to distinguish ITP patients from healthy control

Finally, the investigation of the IgG Levels by Human Platelet Factor-4 and gene expression profiles in pediatric patients with ITP provides substantial insights into the roles of NR1H4 and pf-4 in the pathophysiology of this disorder. Furthermore, the integration of gene expression

profiling into clinical practice could enhance personalized medicine approaches, allowing for tailored treatment options based on individual molecular profiles (Zhang et al., 2020; Hameed et al., 2022). Overall, this study underscores the importance of NR1H4 and Human Platelet Factor-4 as potential biomarkers and therapeutic targets in ITP, (Grace, and Neunert 2016; Provan and Semple, 2022; Mititelu *et al.*, 2024) providing a foundation for future research aimed at unravelling the complexities of this disorder and improving patient outcomes through innovative treatment strategies. The last recommendation to Intensive studies should be conducted on the remaining genes and compared with the incidence rates of the ITP disease to learn more about the gene considered a biomarker for the disease in Iraq and compare the incidence rates of genes causing thrombocytopenia in children.

Conclusion

: The study demonstrated the gene expression of NR1H4 and the pathways by which PF-4 affects immune and platelet functions in ITP. The function of this gene may use a potential biomarker for the diagnosis of ITP. This could pave the way for novel therapeutic approaches ultimately improving management strategies for affected.

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