# Role of Vitamin D Receptor Polymorphism in Newly diagnosed Children with Immune thrombocytopenic Purpura

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## Abstract

**Background:** Vitamin D affects both innate and adaptive immune responses, which have been held responsible in immune thrombocytopenia (ITP) pathogenesis. This study aimed to assess the role of Vitamin D and VDR polymorphism on the pathogenesis of newly diagnosed children with immune thrombocytopenic Purpura.

**Methods:** a case control study was conducted at Hematology and Oncology Unit, Pediatric Department, Zagazig University Hospital on 42 newly diagnosed children with ITP and 42 age and sex-matched healthy children as control group during a period between October2017 until November 2018. All the studied groups subjected to full history taking , thorough clinical examination and laboratory investigation in the form of complete blood count, Bone marrow aspiration (when indicated), serum calcium, serum phosphorus and alkaline phosphatase , serum 25 ( OH) D3 and vit D Cdx-2 (rs11568820)) gene polymorphism was determined using Tetra Amplification refractory mutation system - polymerase chain reaction (T-ARMS-PCR) .

**Results:** vitamin D level of ITP patient was (27.24±14.11 ng/ml) and of the control group (24.24±13.23 ng/ml) with no significant difference between both. There was no significant difference between ITP group and controls regarding allele and genotype distributions of vit D Cdx-2 (rs11568820) (OR 0.92 , 95 % CI 0.42-2.01 and
p=0. 842 for A allele).

**Conclusion:** Vitamin D receptor polymorphism Cdx-2 (rs11568820) does not play a role in the pathogenesis of ITP in this study

**Keywords:** immune thrombocytopenia, polymorphism, vitamin D receptor.

## Introduction

Primary immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterized by an isolated thrombocytopenia (peripheral blood platelet count <100,000) in the absence of other causes.

Immune thrombocytopenic purpura can be clinically classified into three phases Newly diagnosed ITP occurs within 3 months of diagnosis, persistent ITP is present between 3 and12 months post diagnosis and chronic ITP lasting >12 months [1].

The pathophysiology of ITP is complex. The key element in pathogenesis is loss of self-tolerance leading to production of autoantibodies against platelet membrane antigens. The same antibodies may inhibit platelet production in addition activated B cells and increased phagocytic activity and cellular immunity is perturbed. Further, a genetic predisposition to ITP likely plays a role in susceptibility to developing ITP[2,3].

Expression of VDR by immune system cells suggest that vitamin D influences immune system function[3]. More than 30 different human tissues such as the heart, brain, pancreas, stomach, skin, lymphatics, prostate tissue and gonads are composed of cells including T and B lymphocytes that express VDR[5].

The active form of vitamin D (1,25dihydroxy vitamin D) act by binding to vitamin D receptor and mediates its biological activity, vitamin D receptor is located on chromosome 12q13.1, and it has many polymorphic regions [6]. More than 470 common single nucleotide polymorphisms (SNP) have been identified in VDR. These polymorphisms modulate the action of VDR, and their occurrence differs with ethnicity [4].
Vitamin D receptor polymorphisms are associated with the incidence and severity of certain autoimmune diseases [7].
The mechanism by which VDR polymorphism affects autoimmunity is not yet clear, although activation of the receptor contributes to immune responses via regulation of the T-helper (Th)1/Th2 cytokine balance and reduces production of Th2 cytokines [8].
**This study aimed to** assess the role of vitamin D level and VDR polymorphism Cdx-2 (rs11568820) on the pathogenesis of newly diagnosed children with immune thrombocytopenic purpura .

## PATIENTS AND METHODS:

A case control study was conducted at Hematology and Oncology Unit of Pediatric Department and Medical Biochemistry& Molecular Biology department at Zagazig University Hospital during a period between October 2017 until November 2018. The study Included 84 participants divided into two groups; cases group including 42 children with newly diagnosed ITP(thrombocytopenia less than 3months) (26 males and 16 females) with mean age of 5.16±2.97 years and a control group including 42 age and sex-matched healthy children (29 males and 13 females) with mean age of 5.58± 2.5years. The children with persistent or chronic immune thrombocytopenic purpura, and secondary immune thrombocytopenic purpura as collagen disease and malignancy were excluded from the study.
The present study was conducted in accordance with the ethical standards of the Helsinki Declaration of 1964, as revised in 2000, and was approved by the Faculty of Medicine's Research Ethics Committee, University of Zagazig. All participant's parents assigned informed written consent.
All patients included in this study were subjected to full history taking with emphasis on disease duration, history of bleeding (skin, mucus membrane, frank bleeding) , thorough clinical examination including site and shape of bleeding and the following investigations : Complete blood count, Bone marrow aspiration (when indicated), serum calcium, serum phosphorus , alkaline phosphatase and Specific investigations including **Serum 25-hydroxy vitamin D (25(OH) and VDR polymorphisms detection "CDX"**.

### 2.3. Serum 25-hydroxy vitamin D (25(OH):

2 mL venous blood was collected in serum collecting tube to measure Vit D, Genotype by ELISA kit supplied by DRG International, Inc. USA.

### *2.4,. Genotyping of*-VDR polymorphisms detection Cdx-2 (rs11568820).

Genomic DNA was extracted from whole blood using the commercially available G-spin TM Total DNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea). DNA purity and concentration were determined spectrophotometrically at 260 and 280 nm. The purified genomic DNA was stored at −20 °C until use..Tetra Amplification refractory mutation system - polymerase chain reaction (T-ARMS-PCR) assay was used for detection of VDR Cdx-2 (rs11568820)polymorphism as described by of **Fang et al.(9),** using the following primers: Forward outer: G-For (5′ \AGGATAGAGAAAA TAATAGAAAACATT-3′) and A-Rev (5′-ACGTTAAGTTCAGAAAGATTAATTC-3′) and G-Rev (5′-AACCCATAATAAGAAATAA GTTTTTAC-3′) and A-For (5′-TCCTGAGTAAACTAGGTCACAA-3′), cyclic condition was carried out as an initial denaturation at 96°C for 5 minutes; followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 45 seconds; and a final extension at 72°C for 5 minutes. The PCR products were separated by electrophoresis in a 2.5% agarose gel in 0.5× Tris borate EDTA at 120 V for 1 hour.

## STATISTICAL ANALYSIS

The collected data were analyzed by computer using Statistical Package of Social Services version 24 (SPSS), Data were represented in tables and graphs, Continuous Quantitative variables e.g. age were expressed as the mean ± SD & median (range), and categorical qualitative variables were expressed as absolute frequencies (number) & relative frequencies (percentage).

Suitable statistical tests of significance were used after checked for normality. The results were considered statistically significant when the significant probability was less than 0.05 ($P < 0.05$). P-value < 0.001 was considered highly statistically significant (HS), and P-value

## RESULTS:

The study showed that the most predominant Initial bleeding among the studied Immune thrombocytopenic Purpura patients was purpura and ecchymosis (100% & 97.6 %) and only 14.3% of them were presented with epistaxis, bleeding per gum was found in only 7.1% of them. Also, the most predominant treatment modalities among the studied Immune thrombocytopenic Purpura patients was Oral steroids (47.6 %), followed by IVIG in 40.47% and only 11.9% of them were treated with Dexamethasone IV. **Table (1)**

**The table 2** showed that mean WBCs among the studied thrombocytopenic Purpura patients was 8.99 ± 3.84, and it was 8.37±1.92 among health control with no statistically significant difference between ITP and healthy control regarding WBCs and Hemoglobin level, but Platelet count among ITP was statistically lower than healthy control.

This study showed that more than 2/3 of the studied Immune thrombocytopenic Purpura patients were GG genotype (69.0%) also 66.7% of healthy control were GG genotype, with no significant difference between groups. About 82.2% of the studied patients and 80.9% of healthy control had G alleles, with no significant difference between groups **Table (3)**

The study showed that mean vitamin D level of ITP group was 27.24±14.11 ng/ml, with no statistically significant difference. Also there was no significant difference between both groups regarding vitamin D level and status **Table 4** .

The study showed that there was no significant relation between VDR gene polymorphism and vitamin D level and status among the ITP patients **table 5**.

This study showed that mean age of ITP cases with the GG genotype of VDR polymorphism group is 5.17±3.05 years old, with a range from 2 to 10 years old and there was no significant relation between VDR polymorphism regarding sex and age of studied group .Although th**e** mean platelet count of ITP cases with GG genotype , was decreased compared to other genotypes ,There was no significant relation between VDR gene polymorphism and CBC findings of studied cases . **Table 6**

**Table (1) Initial bleeding and treatment modalities of the studied cases**

| Item | ITP cases (N=42) | |
|---|---|---|
| | No. | % |
| **Skin** | | |
| Purpuric | **42** | **100.0** |
| Ecchymosis | **41** | **97.6** |
| **mucous membranes** | | |
| Epistaxis | **6** | **14.3** |
| Bleeding per gum | **3** | **7.1** |
| GIT bleeding | **0** | **0** |
| Urinary | **0** | **0** |
| **Serious Bleeding** | | |
| **ICH** | **0** | **0** |
| **Treatment modalities** | | |
| Oral steroids | **20** | **47.6** |
| IVIG | **17** | **40.47** |
| Dexamethasone IV | **5** | **11.90** |

**Table (2) Hematological data of the studied groups**

| Hematological findings | ITP cases (N=42) | Control(N=42) | MWT | p-value |
|---|---|---|---|---|
| **Platelet count (c/mm)** | | | | |
| Mean ± SD ▪ | 22.12 ±13.14 | 171.88 ± 46.57 | 7.000 | 0.000* (HS) |
| Median (Range) ▪ | 19.5(1-60) | 157(150-298) | | |
| **Hemoglobin(gm/dl** | | | | |
| Mean ± SD ▪ | 10.95±1.46 | 11.27±0.72 | 827.000 | 0.622 (NS) |
| Median (Range) ▪ | 11.25(7.3-14) | 11.35(10-12.5) | | |
| **WBCs (c/mm)** | | | | |
| Mean ± SD ▪ | 8.99±3.84 | 8.37±1.92 | 870.000 | 0.914 (NS) |
| Median (Range) ▪ | 8.25(4.3-18.7) | 8.1(4.9-12.3) | | |

**Table (3) Distribution of Vitamin D Receptor Polymorphism &alleles among the studied groups**

| Vitamin D Receptor Polymorphism | ITP cases (N=42) | | Control(N=42) | | Test | P- value |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | | |
| **AA** • | 2 | 4.8 | 2 | 4.8 | 0.061 | 0.970 (NS) |
| **GA** • | 11 | 26.2 | 12 | 28.6 | | |
| **GG** • | 29 | 69.0 | 28 | 66.7 | | |
| **Vitamin D Receptor alleles** | **ITP cases** | | **Control** | | **OR(95%C.I)** | **P- value** |
| | **No.** | **%** | **No.** | **%** | | |
| **A** • | 15 | 17.86 | 16 | 19.05 | 0.92(0.42-2.01) | 0.842(NS) |
| **G** • | 69 | 82.14 | 68 | 80.95 | 1.08(0.49-2.36) | 0.843(NS) |

**Table (4) Vitamin D level and status among the studied groups**

| Item | ITP (N=42) | | Control(N=42) | | Test | P- value |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | | |
| **Vitamin D level ng\ml** | | | | | | |
| Mean ± SD | 27.24±14.11 | | 24.24±13.23 | | #744.50 | 0.218 (NS) |
| Median (Range) | 28(4-50) | | 28(5-44) | | | |
| **Vitamin D status** | | | | | | |
| Deficiency (0-20) | 17 | 40.5 | 17 | 40.5 | ◊0.104 | 0.949 (NS) |
| Insufficiency (20-30) | 7 | 16.7 | 6 | 14.3 | | |
| Sufficiency (30-50) | 18 | 42.9 | 19 | 45.2 | | |

**Table (5) Relation between vitamin D level and status among the studied ITP children and VDR polymorphism .**

| Item | VDR polymorphism | | | | | | Test | P-value |
|---|---|---|---|---|---|---|---|---|
| | AA (N=2) | | GA (N=11) | | GG (N=29) | | | |
| | No. | % | No. | % | No. | % | | |
| **Vitamin D level (ng/ml )** | | | | | | | | |
| Mean ± SD | 26.5±19.09 | | 31.27±15.38 | | 25.76±13.61 | | #1.588 | 0.452 (NS) |
| Median (Range) | 26.5(13-40) | | 38(10-49) | | 27(4-50) | | | |
| **Vitamin D status** | | | | | | | | |
| Deficiency (0-20) | 1 | 50.0 | 5 | 45.5 | 11 | 37.9 | *3.853 | 0.429 (NS) |
| Insufficiency (20-30) | 0 | 0.0 | 0 | 0.0 | 7 | 24.1 | | |
| Sufficiency (30-50) | 1 | 50.0 | 6 | 54.5 | 11 | 37.9 | | |

**Table (6) Relation between demographic data &complete Blood picture of the studied ITP children and VDR polymorphism**

| Demographic data | AA (N=2) | | GA (N=11) | | GG (N=29) | | Test | P-value |
|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | | |
| **Sex** | | | | | | | | |
| Male | **0** | **0.0** | **8** | **72.7** | **18** | **62.1** | ***3.797** | **0.150 (NS)** |
| Female | **2** | **100.0** | **13** | **27.3** | **11** | **37.9** | | |
| **Age (years)** | | | | | | | | |
| Mean ± SD | **7.0 ± 4.24** | | **4.5 ± 2.36** | | **5.17±3.05** | | **#0.999** | **0.607 (NS)** |
| Median (Range) | **7(4-10)** | | **4(2-10)** | | **4(2 -10)** | | | |
| **Complete Blood picture** | | | | | | | | |
| **Platelet count (c/mm)** | | | | | | | | |
| Mean ± SD | 28.5 ±19.09 | | 15.73±5.9 | | 24.1±14.29 | | 3.92 | 0.140 (NS) |
| Median (Range) | 28.5(15-42) | | 13(10-28) | | 20(1-60) | | | |
| **Hemoglobin (gm/dl)** | | | | | | | | |
| Mean ± SD | 11.0 ±0.98 | | 10.39±1.81 | | 11.16±1.33 | | 1.894 | (NS) |
| Median (Range) | 11(10.3-11.7) | | 10.5(7.3-12.7) | | 11.4(8.4-14) | | | |
| **WBCs (c/mm)** | | | | | | | | |
| Mean ± SD | 6.05 ±0.49 | | 9.66±4.01 | | 8.94±3.88 | | 2.464 | 0.292 (NS) |
| Median (Range) | 6.05(5.7-6.4) | | 9(4.3-17.3) | | 8(4.5-18.7) | | | |

**DISCUSSION:**

Childhood immune thrombocytopenic purpura (ITP) is one of the most common benign hematologic disorders. It is characterized by isolated, immune-mediated thrombocytopenia. The etiology of ITP is unclear but is likely due to genetic or acquired factors (4)Vitamin D has an important influence on the host's immune system by modulating both innate and adaptive immunity and regulating the inflammatory cascade (11).

Many studies found that Vitamin D receptor polymorphisms are associated with the incidence and severity of certain autoimmune diseases. So our study aimed to assess the role of Vitamin D and VDR polymorphism on the pathogenesis of newly diagnosed children with immune thrombocytopenic Purpura.

Our results showed that the most predominant Initial bleeding among the studied Immune thrombocytopenic Purpura patients was purpura and ecchymosis (100% & 97.6 %) and only 14.3% of them were presented with epistaxis, bleeding per gum was found in only 7.1% of them

Our results were in agreement with Evim et al.(10) ,who reported in their study on 201 children with ITP by using the new definitions of the International Working Group (IWG) on ITP, that the most frequent symptoms were petechia and ecchymosis (71%). Thirty-six children (18%) were admitted with epistaxis and/or gum bleeding along with petechia and ecchymosis. Twenty-three patients (11%) had no bleeding manifestations.

Also our data was in agreement with what was reported by Saeidi et al.(11), that the frequency of initial symptoms including petechiae and ecchymosis was 60.5% and 61%, respectively in all patients, but severe bleeding rarely occurred.

Also, Osman,2012 reported in their review article that the disease onset is abrupt with bruises and petechial rashes affecting almost all patients. Epistaxis may occur in about one third of patients and hematuria is uncommon. (12)

Regarding therapy given to our patients, 20 patient (47.6% ) received oral steroids, 17 patients (40.47%) received intravenous methyl prednisolone and 5 patient (11.90 %) received immunoglobulin (IVIG).

In the study done by Evim et al., (2014), IVIG was administered to 66 (65%) children, whereas 36 (35%) received corticosteroids as the first therapeutic choice. (10)

While in the study done by Saeidi et al., (2014), 86 patients (26.6%) received only methylprednisolone, 15 (4.6%) received IVIG, 207 (64.1%) received both drugs and 15 (4.6%) received none. (11)

Grimaldi-Bensouda et al., (2017) reported in their study on 257 children aged 6 months–18 years and diagnosed with primary ITP, 208 (80.9%) patients were started initial treatment while the other 49 (19.1%) were undergo watchful waiting strategy. Of the 208 patients, 63/208(30.3%) were initially treated with Corticosteroids alone, 99/208 (47.6%) were initially treated with Intravenous immunoglobulin (IVIG) alone while 46/208 (22.1%) were initially treated with Corticosteroids and IVIG. (17)

This study showed no statistically significant difference between ITP and healthy control regarding WBCs and Hemoglobin level, but Platelet count among ITP cases was statistically lower than healthy control.

All studies and guidelines diagnose ITP as isolated thrombocytopenia with an otherwise normal complete blood count. Anemia from blood loss may be present, but it should be proportional to the amount, and the duration, of bleeding and may result in iron deficiency. If anemia is found, the reticulocyte count may help define whether it the result of poor production or increased destruction of red blood cells (RBCs) (Provan et al., 2010; Neunert et al., 2011; Osman, 2012)(15).

Our results showed that the mean vitamin D level of ITP patient (27.24±14.11) and of the control group (24.24±13.23) with a statistical non significant difference between both groups (p-value:0.218).

Čulić et al., (2016) reported in their study on 21 child who were recruited from both chronic ITP patients and newly diagnosed ITP, that VD deficiency (values <75 nmol/L) was detected in 11 patients with newly diagnosed ITP, and seven patients with chronic ITP. Only three patients with newly diagnosed, and none with chronic ITP had normal VD values. Newly diagnosed ITP patients had statistically significantly higher values (P <.044) of VD than the patients with chronic type of ITP. (17)

The Cdx-2 polymorphism is located in the promoter region of VDR, and it carries a G to A sequence change that affects the function of the transcription factor. The presence of the G allele at the Cdx-2-binding site reduces the transcriptional activity of VDR promoter by 70% versus the A allele [14, 15] .

Regarding the three Cdx-2 genotype distribution in our participants, our results showed that the most presented genotype in the in the case group GG presented in 29patient (69 %) and GA genotypes were presented in 11 patient (26.2%) cases for each and the AA genotype was present in 2patient (4.8%) cases.

Our results showed that in control group the A allele was presented in 16 (19.05%) of them and the G allele was presented in 68 (80.95%) subjects. While in case group the A allele was presented in 15 (17.86%) cases and the G allele was presented in 69 (82.14%) of cases. There was a statistical non significant difference between both groups with p-value: 0.842and 0.843 respectively.

In the study done by Yesil et al., (2017) they reported that the distribution of the three genotype groups (GG, GA, AA) was significantly different between the ITP patients and controls (P = 0.025). And also reported that the GG genotype of Cdx-2 was overrepresented in the ITP group (84.1%) versus controls (62%). And found the frequency of the Cdx-2 A allele was significantly different between ITP patients and controls and no significant differences were observed between ITP patients and controls of frequency of the Cdx-2 G allele and concluded that the Cdx-2 A allele was associated with a decreased risk in the ITP (OR, 0.343; 95% CI: 0.150–0.782). (4)

Regarding the frequency of the Cdx-2 alleles in our studied groups, our results showed that in control group the A allele was presented in 16 (19.05%) of them and the G allele was presented in 68 (80.95%) subjects. While in case group the A allele was presented in 15 (17.86%) cases and the G allele was presented in 69 (82.14%) of cases. There was a statistical non-significant difference between both groups with p-value: 0.842and 0.843 respectively.

Regarding serum Vitamin D level in the different Cdx-2 genotypes in cases group, our results showed that there was no significant difference between the three genotypes with p-value: 0.452 . In agreement with our findings, Yesil et al., (2017) reported that the mean serum 25(OH)D in ITP patients with the Cdx-2 A allele (20.8 ± 8.72 ng/mL; range, 11.5–37 ng/mL) was not significantly different from that in ITP patients who did not have the Cdx-2 A allele (18.68 ± 9.18 ng/mL; range, 4.4–46.2 ng/mL; P = 0.576. (4)

Also , this study showed that mean age of ITP cases with the GG genotype of VDR polymorphism group was 5.17±3.05 years old, with a range from 2 to 10 years old. There was no significant relation between VDR polymorphism regarding sex and age of studied group . Also, our results showed that mean platelet count of ITP cases with GA genotype was 29.27±19.16 while it was 12.83±13.15 in cases with GG with no significant differences .

## CONCLUSION:

Vitamin D receptor polymorphism doesn't play a role in the pathogenesis of newly diagnosed children with immune thrombocytopenic Purpura.

## LIMITATION OF STUDY:

The conclusions of this study were limited by the small number of patients .

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