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Evaluation of T helper17 as skeletal homeostasis factor in peripheral blood mononuclear cells and T helper cells of end-stage renal disease cases with impaired parathyroid hormone

Running title: *T helper17 cells in end stage renal diseases* with impaired **parathyroid hormone**

Roza Motavalli [1,2], Mohammad Sadegh Soltani-Zangbar [3,4], Khadijeh fereydoonzadeh [5], Mahsa Hajivalili [6], Javad Ahmadian Heris [7], Houman Kahroba [8,9], Bahram Niknafs [5], Farhad Motavalli Khiavi [10], Sanam Dolati [11], Mohammadreza Sadeghi [1], Mehdi Yousefi [2,4*], Jalal Etemadi [2*]

1. *Department of Molecular Medicine, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran.*
2. *Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
3. *Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.*
4. *Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.*
5. *Kidney Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
6. *Department of Immunology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
7. *Department of Allergy and Clinical Immunology, Pediatric Hospital, Tabriz University of Medical Sciences, Tabriz, Iran.*
8. *Department of Toxicogenomics, GROW School for Oncology and Developmental Biology, Maastricht University, the Netherlands.*
9. *Center for Environmental Sciences, University of Hasselt, Agoralaan D, BE-3590, Hasselt, Belgium.*
10. *Department of Virology, Pasteur Institute of Iran, Tehran, Iran.*
11. *Physical Medicine and Rehabilitation Research Center, Aging Research Institute, Tabriz University of Medical Sciences, Tabriz, Iran.*

***Corresponding Authors:**

*Corresponding Authors:
Mehdi Yousefi, PhD, Associate Professor
Department of Immunology, School of Medicine,
Tabriz University of Medical Sciences, Tabriz, Iran
Tel: +98-4113364665; Fax: +98-4113364665; Email: yousefime@tbzmed.ac.ir

Jalal Etemadi, MD, Associate Professor of Nephrology,
Department of Nephrology, Tabriz University of Medical Sciences, Tabriz, Iran
Tel: 0098 41 33373966; Fax: 0098 41 33373966
PO Box: 51666114756, Email: etemadij@tbzmed.ac.ir

Abstract

Background: Chronic renal failure is mainly connected with high and low parathyroid hormone (PTH) levels and immunological impairments. The present study aimed to evaluate T helper 17 (Th17) cells as a crucial modulator of the immune system and skeletal homeostasis in hemodialysis patients with impaired intact PTH (iPTH).

Methods: In this research, blood samples were taken from ESRD patients with high (> 300 pg/mL), normal (150-300 pg/mL), and low (< 150 pg/mL) serum intact parathyroid hormone (iPTH) levels (n = 30 in each group). The frequency of Th17 ($CD4^+$ $IL17^+$) cells was evaluated by flow cytometry in each group. The expression levels of Th17 cell-related master transcription factors, cytokines in peripheral blood mononuclear cells (PBMC), and Th cells, and the level of the mentioned cytokines were determined in the supernatant of PBMCs.

Results: The number of Th17 cells remarkably increased in subjects with high iPTH against low and normal iPTH. Also, RORɣt and STAT3 levels were significantly higher in high iPTH ESRD patients than in other groups in the expression of mRNA and protein levels. These findings are confirmed by evaluating the IL-17 and IL-23 in the supernatant of cultured PBMCs and isolated Th cells.

Conclusion: Our findings indicated that increased serum PTH levels in hemodialysis cases may be involved in increasing the differentiation of CD4+ cells to Th17 cells in PBMC.

Keywords: Chronic kidney disease; IPTH; Th17; STAT3; RORɣt

1. Introduction

End-stage renal disease (ESRD) is defined as a constant decrease in renal function, which can be severely lethal in the absence of hemodialysis or transplantation (1). One of the main problems in ESRD is related to immune dysregulation leading to increased susceptibility to infections, which may lead to even early death in cases (2-4).

Most epidemiologic studies have indicated that parathyroid hormone (PTH) abnormalities are extremely common in ESRD cases and associated with immunologic dysfunction. Beyond the role of PTH in chronic kidney disease-mineral bone disorder, more attention has been paid to understanding the pro-inflammatory role of PTH (5). Recent studies support this theory that increased or decreased levels of PTH likely are involved in the impairment of the humoral and cellular responses. This effect of PTH has been attributed to the PTH receptor, which is expressed in various immune cells (5, 6).

Recently, it has been shown that cPTH infusion in mice stimulates the differentiation of $CD4^+$T cells into Th17 by a TNF-related network. In addition, these 17 cells are also considered pro-osteoclastogenic subsets of $CD4^+$ T cells determined by their ability to express IL-17A.(7). To the best of our knowledge, there are no studies about the relation of PTH with Th17 cells in ESRD patients.

Regarding the pro-osteoclastogenic and pro-inflammatory function of IL-17A, we evaluated the Th17 cells, as a signature subset of $CD4^+$T cells and prominent IL-17 producer cells, in PBMCs of ESRD patients with low, normal, and high iPTH levels.

78 ## 2. Materials & Methods

79 ### *2.1. Study population & blood sampling*

80 The research population included end-stage renal disease (ESRD) patients who were referred to
81 the hemodialysis ward of Imam Reza hospital from May to June, 2021 and were under
82 hemodialysis treatment for at least three months. The acceptable age range was 30 to 85 years old.
83 The study was approved by the Tabriz University of Medical Sciences ethics committee
84 (IR.TBZMED.REC.1398.1155), and written informed consent was taken from all participants
85 before enrollment in the study. The medical history of the subjects was obtained from medical
86 records. Exclusion criteria were active infection, active malignancy, underlying rheumatologic
87 diseases, history of renal transplantation, immunosuppressive drug usage, history of
88 parathyroidectomy, high serum ferritin level, and receiving calcium-sensing receptor antagonist
89 (cinacalcet). Finally, 90 HD patients were selected for further assessment according to the
90 mentioned criteria.

91 In the next step, 10 ml of peripheral blood was obtained from each patient, Out of which 2 mL was
92 separated for detecting Fe, total iron-binding capacity (TIBC), Ferritin, vitamin D, and IPTH in
93 serum, and the remaining 8 ml was processed for peripheral mononuclear cells separation by
94 FicollHypaque (Sigma, USA) gradient density. The isolated enriched PBMCs were washed twice
95 with Phosphate-buffered saline (PBS). They were re-suspended at 2×10^6 cells/mL in Roswell Park
96 Memorial Institute (RPMI) 1640 medium with 100 U/mL penicillin and 100 mg/mL streptomycin
97 plus 10% fetal bovine serum (Gibco, USA).

98 ### *2.2.Flow cytometry of the Th17 cells population*

99 Th17 frequency was evaluated by stimulating the harvested PBMCs from each sample with
100 Phorbol Myristate Acetate (PMA) with a concentration of 10 ng/mL and ionomycin (0.5 μm).

Next, intracellular cytokine transport inhibitor Golgi Stop (1 µl/mL, BD biosciences, USA) was added to the sample. Afterward, the cells were stained with FITC conjugated anti-CD4 (BD biosciences, USA) at 4°C for 20 min. After fixation and permeabilization, PE-labeled anti-IL-17 (BD biosciences, USA) was added according to the operating instructions (8). Finally, antibody-specific Isotype controls were applied to confirm the results (BD biosciences, USA).

2.3. Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) for relative gene expression of STAT3 and ROR-γt

Total RNA extraction was performed using an RNA extraction kit (SinaClon, Tehran, Iran) according to the manufacturer's instructions. The isolated RNA was quantified by spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc., Wilmington, USA). The c-DNA was first synthesized with a c-DNA synthesis kit (Thermo Fisher, Waltham, MA) in 20 µl reaction volume with Eppendorf Mastercycle EP Gradient thermal cycler. Next, it was subjected to RT-qPCR (Rotorgene, Qiagen, Germany) by applying master-mix SYBR green (Ampliqon A/S, Denmark) and the primer sets of each target gene as follows: *STAT3* forward 5′CTTTGAGACCGAGGTGTATCACC3′ and reverse 5′GGTCAGCATGTTGTACCACAGG3′; *ROR-γt* forward 5′GAGGAAGTGACTGGCTACCAGA3′ and reverse 5′GCACAATCTGGTCATTCTGGCAG3′. Moreover, 5′CACCATTGGCAATGAGCGGTTC3′ forward and 5′AGGTCTTTGCGGATGTCCACGT3′ reverse were used for *β-actin* as the housekeeping gene (9). Relative gene expression was evaluated by the $2^{-\Delta\Delta ct}$ method.

2.4. Detecting IL-17 and IL-23 levels by enzyme-linked immunosorbent assay (ELISA)

IL-17 and IL-23 concentrations were measured with human IL-17 and IL-23 ELISA kits (MyBioSource, San Diego, CA, USA). The cytokine level of each sample was measured in triplicate.

2.5. STAT3 Western Blotting assay

Total protein was extracted from lysed PBMCs, and T cells were isolated. The protein concentration of each sample was measured by the BCA assay kit (Merck, Germany). An equal amount of protein was separated from all samples by the SDS-PAGE method. Afterward, samples were electro-transferred to nitrocellulose membranes. For blocking, 5% skimmed milk solution (1X TBST) was applied for 1 hour at room temperature (RT), followed by overnight incubation at 4°C. Mouse anti-human STAT-3 (0.1 µg/mL, R&D systems) were used as specific primary antibodies. The next day, membranes were washed three times with 1xTBST and then underwent 1 h of incubation with a secondary antibody at RT. After that, membranes were washed and used for visualization (10).

2.6. Magnetic-activated cell sorting (MACS)

CD3 + T cells were isolated using the MACS technique with a negative selection protocol (Miltenyi Biotec, San Diego). Phosphate-buffered saline (PBS) (Sigma-Aldrich, Germany) was used for rinsing isolated cells. Next, 5 mL of supplemented medium with heat-inactivated fetal calf serum 10 % (FBS), penicillin (100 U/mL), L-glutamine (200 mM), and phorbol myristate acetate (10 ng/mL) (PMA; eBioscience, San Diego, CA) were used to cultivate CD3 + T cells and incubate them for 48 h at 37°C and 5% CO_2. The cell culture supernatant was evaluated for detecting cytokine levels in CD3 + T cells by enzyme-linked immunosorbent assay (ELISA). Also, real-time polymerase chain reaction (RT-PCR) and western blotting analysis were done on separated cells.

2.7. ***Statistical analysis***

After the Kolmogorov-Smirnov normality check for data distribution, the analysis of variance (ANOVA) test followed by Dunnett's T3 multiple comparisons post-huck was used for data

147 evaluation. Variables were expressed as mean ± standard deviation, with $P < 0.05$ as the
148 significance level. Furthermore, SPSS software version 23 was used for all statistical analyses.

2. 3. Results

3.1. General aspects of the study population

The recruited ESRD patients were divided into three groups based on their serum iPTH levels according to the K/DOQI guidelines on bone and mineral metabolism. The first group (n = 30) included patients with high iPTH levels in serum (> 300 ng/mL). The second group (n = 30) included patients with normal iPTH levels (150-300 ng/mL). The acceptable target range for serum parathyroid hormone (PTH) in dialysis patients varied from 150 to 300 pg/mL in the KDOQI guidelines (11). The third group (n = 30) included patients with low iPTH levels (150 ng/mL).

The mean age range for each group was 60.45 ± 14.89, 61.50 ± 14.17, and 52.75 ± 10.25, respectively. No significant statistical differences were observed within the groups for other laboratory parameters. In all groups, most patients received three sessions of HD per week. The detailed demographic aspects of patients are presented in Table 1.

3.2.The frequency of circulating Th17 cells in the peripheral blood of the studied group

As shown in Fig. 1, there was no remarkable difference in the Th17 population between normal iPTH and low iPTH patients. Meanwhile, Th17 cells were considerably elevated in high iPTH patients compared to the normal iPTH group (P = 0.0132). Also, significant variation was detected between high iPTH and low iPTH in HD patients (P = 0.0078).

3.3. The mRNA expression level of master Th17-related transcription factors

The relationship between the PTH levels and Th17 cell variation was determined by evaluating the mRNA expression levels of STAT3 and RORγt in PBMC (Fig. 2) and T cells population (Supplementary Fig. 1). The results showed no significant difference between the normal iPTH and low iPTH cases in RORγt and STAT3 expressions. However, a remarkable difference was observed between the high and normal iPTH groups in STAT3 (P = 0.0236) and RORγt (P =

0.0387) and also with the low iPTH group in STAT3 (P = 0.0012) and RORγt (P = 0.0001) in PBMC. In addition, these results were confirmed by evaluating the factors mentioned in the T cells population, presented in Supplementary Fig. 1 and Table 1.

3.4. Detection of Th17-related transcription protein level

The expression of STAT3 at the protein level was evaluated by western blotting in PBMCs (Fig. 3) and T cells population (Supplementary Fig. 2). The results showed no significant change between the low iPTH and normal iPTH in HD patients. However, a significant increase was demonstrated in the high iPTH group compared to the low (P = 0.0053) and normal iPTH groups (P = 0.0010). These results were confirmed by evaluating the mentioned proteins in the T cells population (Supplementary Fig. 2). The western blot test was performed in triplicate.

3.5. Levels of Th17-related cytokines in studied cases

The relation between the PTH levels and Th17-associated cytokines was identified by evaluating the supernatant of cultured PBMCs and T cells population (Supplementary Fig. 3) by ELISA. As indicated in Fig. 4 and Table 1, higher IL-17 and Il-23 levels were detected in HD patients with high iPTH compared to normal (P = 0.346; P = 0.0232) and low iPTH groups (P = 0.0059; P = 0.0087), respectively. Also, evaluation the supernatant of the cultured T cells population verified these results (Supplementary Fig. 3 and Table 2). Finally, no considerable variation was detected in the mentioned cytokines between the low iPTH and normal iPTH groups in HD patients.

4. Discussion

Our study revealed an increase in the peripheral Th17 cell population, STAT3, RORγt, IL-17, and IL-23 of high iPTH cases compared to normal iPTH and low iPTH groups who were under hemodialysis procedure. In addition, no change was detected in comparison between the low iPTH and normal iPTH groups.

PTH is assessed as a major target of chronic kidney disease-mineral bone disorder (CKD-MBD) in dialysis cases. In this regard, dysregulated hormone levels and other uremic toxins promote immunological defects in the mentioned cases (5, 6, 12). According to some studies that have confirmed the expression of PTH receptors in the lymphocytes, two signaling pathways are considered for the interactions of PTH-PTH receptors on the T cells. First, PTH may induce an adenylate cyclase system in the lymphocytes and develop cAMP. Next, the interaction of PTH-PTH receptors affects the turnover of phospholipids in several tissues. The mentioned effects can develop diacylglycerol, which stimulates protein kinase C (13).

Former in vitro and in vivo research studies verified that PTH could stimulate the T cell expansion in normal volunteers due to its ability to increase the conduction of calcium into its target cells, leading to the interleukin-2 production and stimulation of protein kinase C (13-15).

Griveas et al. observed that increased PTH affects lymphocyte function leading to the variation of cellular immunity in a peritoneal dialysis individual (16). Ozdemir et al. reported that the ratio of CD4+/CD8+ lymphocytes decreased in ESRD patients with a low level of iPTH, causing immune defects and increased susceptibility to infections (17). Other studies reported that low levels of PTH can be considered a risk factor for infection-associated mortality in dialysis patients and peritoneal dialysis cases. Besides, the raised levels of PTH had the lowest infection rates while having a potential role in decreasing bone density (5, 18).

In line with previous findings, two studies have shown that parathyroidectomy can lead to the repair of the impaired proliferation of T cells in HD patients with increased PTH levels. The significant decrease in PTH due to parathyroidectomy will likely exert a beneficial effect on humoral immunity balance (19, 20).

Osteoimmunology is a scientific discipline that studies the interactions between the bone and the immune system. In this respect, T cells in the immune system have a significant role in controlling the skeleton in cases of diseases. Over the recent decades, it has been established that the number of circulating T cell population declines in HD patients accompanied by a decreased proliferative response and increased apoptosis when stimulated by various antigens continuously (3, 21, 22). In this context, Th17/Treg balance has been considered a prominent factor for the induction or regulation of inflammation in HD cases (23-25). A study from China illustrated that alteration in the frequency of Th17 cells is affected by the severity of calcification, uremia independence of dialysis membrane type, and dialysate in HD cases (26). The Th17 subset, a population of CD4+ groups, displays osteoclastogenic activity and skeletal homeostasis. PTH induces the release of TNF from immune cells that cause CD4+ differentiation into Th17 cells and subsequent production of IL-17, RANKL, TNF, IL-1, and IL-6. Together with low levels of IFNγ, these proteins contribute to bone loss (27-29). In the bone marrow, IL-17 promotes the development of RANKL, overexpresses the RANKL receptor, and induces bone resorption. In addition, IL-17 blunts bone formation by suppressing the Wnt signaling pathways in osteoblasts (29, 30). Therefore, Th17 cells are targeted by PTH and play a role in PTH-induced bone resorption.

Consistent with previously published studies (26), our findings indicated that the differentiation of the Th17 population was more significant in high iPTH of HD cases than in other groups. Hyper iPTH might playe a potential role in the expansion of the Th17 population in HD patients confirmed via elevated Th17 cell-related prominent transcription factors and cytokines. A positive correlation between PTH levels and Th17 populations in several studies indicated that phosphate

and consequence PTH levels might be an essential factor for predicting Th17 cell differentiation in HD patients (31). Osteoblasts and osteoblasts are not the only cells that mediate PTH function, Th17 cells and IL-17 are also included in this pathway. Accordingly, targeting Th17 is likely to be one of the therapeutic strategies for the treatment of hyperparathyroidism-induced bone loss in hemodialysis patients.

Further evaluations are needed in this regard because this study has some limitations, such as a small number of included cases with various durations of hemodialysis coupled with the lack of assessments on other immune cells and other factors followed by comparing bone density and infection among groups. Our study is following up to collect more data and evaluate more immune factors related to PTH, which will cause more solid conclusions.

Conclusion

Our study showed an increase in the Th17 cell population in PBMC of high iPTH cases compared to normal iPTH and low iPTH groups under the hemodialysis procedure. It can be concluded that PTH may be involved in cytokine production in bone and increase the differentiation of CD4+ into Th17 cells along with the production of IL-17. Nevertheless, further studies are needed to confirm these results. The obtained data may expand proper horizons for the treatment or the prevention of bone loss problems and serious infections in hemodialysis patients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table_1: Demographical and immunological features of hemodialysis patients with different iPTH ranges.

Variant		<150 mean±SD (N=30)	150<>300 mean±SD (N=30)	>300 mean±SD (N=30)	p Value		
					<150 vs 150<>300	<150 vs >300	150<>300 vs >300
Age		60.45±14.89	61.50±14.17	52.75±10.25	NS	NS	NS
Vitamin D (mg/dl)		45.95±16.83	51.45±19.09	49.10±17.82	NS	NS	NS
Time of dialysis (month)		30.00±30.71	42.20±35.45	31.25±47.30	NS	NS	NS
Ferritin (µg/l)		224.8±157.4	188.3±64.16	260.3±166.5	NS	NS	NS
Fe (%)		64.00±60.09	58.68±17.57	65.16±26.22	NS	NS	NS
TIBC (µmol/l)		320.0±63.64	316.1±109.5	311.3±57.03	NS	NS	NS
Hemodialysis Type	Fistol	16	21	21	NS		
	Kateter	14	9	9			
STAT-3 Relative Gene Expression		1.000±0.1014	1.084±0.2529	1.679±0.6942	NS	0.0012	0.0236
RORγT Relative Gene Expression		0.999±0.0751	1.105±0.1945	1.480±0.5229	NS	0.0001	0.0387
STAT-3 Protein Expression%		30.00±7.434	28.45±7.280	41.55±12.96	NS	0.0053	0.0010
Th17 Lymphocytes%		4.155±1.390	4.248±1.481	5.577±2.326	NS	0.0078	0.0132
IL-17 Secretion (pg/ml)		32.67±11.38	34.70±13.25	43.93±15.34	NS	0.0059	0.0346
IL-23 Secretion (pg/ml)		40.38±13.06	41.47±14.14	53.73±18.14	NS	0.0087	0.0232

TIBC: Total iron-binding capacity; <150: Hemodialysis patients with under 150 IPTH; 150<>300: Hemodialysis patients with between 150 and 300 IPTH; >300: Hemodialysis patients with more than 300 IPTH. $p < 0.05$ was considered as significant.

Figure legends

Fig. 1. Th17 frequency in ESRD patients with low, normal, and high iPTH levels. The plots of $TCD4^{+}IL\text{-}17^{+}$ cells indicate of Th17 population in ESRD patients with different amounts of iPTH (n = 30). The percentage of Th17 cells showed significant elevation in high iPTH (> 300) compared to the normal PTH (> 150 and 300 <) (P = 0.0132) and low patients (150 >) (P = 0.0078). Isotype controls were applied for corrected gating. The achieved data were expressed as mean ± SD. P < 0.05 was considered statistically significant (Th17: T-helper type 17; PTH: Parathyroid hormone; ESRD: End-stage renal disease).

Fig. 2. The Expression level of Th17 -associated transcription factors in ESRD cases with different iPTH levels. RORyt and STAT3 levels indicated a significant increase in high iPTH (> 300) compared to the normal (> 150 and 300 <) (P = 0.0387; P = 0.236) and low patients (150 >) (P = 0.0001; 0.0012) respectively. β actin was used for the normalization of mRNA. The data are expressed as mean ± SD. P< 0.05 was considered statistically significant (RORγt: receptor-related orphan nuclear receptor γt; STAT3: signal transducer and activator of transcription 3; Th17: T-helper type 17; PTH: Parathyroid hormone; ESRD: End-stage renal disease)

Fig. 3. Western blot analysis of STAT3 protein in PBMCs of patients with ESRD with different iPTH levels. STAT3 levels indicated a significant increase in high iPTH (> 300) in comparison to the normal PTH ((> 150 and 300 <) (P = 0.0010) and hypoparathyroidism patients (150 >) (P = 0.0053). Results are expressed as mean ± SD. P < 0.05 was considered statistically significant (PBMCs: peripheral blood mononuclear cells; STAT3: signal transducer and activator of transcription 3; PTH: Parathyroid hormone; ESRD: End-stage renal disease).

Fig. 4. Th17 cell-associated cytokines evaluation in the supernatant of cultured. The concentrations of IL-17 and IL-23 were remarkably enhanced in high iPTH cases (> 300) in comparison to the control ((> 150 and 300 <) (P = 0.0346; P = 0.232) and with low iPTH patients (150 >) (P = 0.0059; P = 0.0087), respectively. Data are expressed as mean ± SD. P < 0.05 was considered statistically significant (IL: interleukin, PBMCs: peripheral blood mononuclear cells, Parathyroid hormone, ESRD: End-stage renal disease).

Supplementary Table_1: Isolated T cell characteristics of hemodialysis patients with different iPTH ranges.

Variant	<150 mean±SD (N=30)	150<>300 mean±SD (N=30)	>300 mean±SD (N=30)	p Value		
				<150 vs 150<>300	<150 vs >300	150<>300 vs >300
STAT-3 Relative Gene Expression	1.000±0.08663	1.131±0.3019	1.812±0.6068	NS	<0.0001	0.0017
RORγT Relative Gene Expression	0.999±0.08201	1.196±0.3391	1.823±0.9017	NS	<0.0001	0.0262
STAT-3 Protein Expression%	31.25±9.159	30.40±8.617	39.35±10.73	NS	0.0255	0.0211
IL-17 Secretion (pg/ml)	20.17±11.68	21.33±11.79	31.80±15.98	NS	0.0071	0.0186
IL-23 Secretion (pg/ml)	26.95±12.09	28.97±14.70	42.73±16.65	NS	0.0006	0.0050

<150: Hemodialysis patients with under 150 IPTH; 150<>300: Hemodialysis patients with between 150 and 300 IPTH; >300: Hemodialysis patients with more than 300 IPTH. $p < 0.05$ was considered as significant.

Supplementary Fig. 1. The Expression level of Th17 -associated transcription factors in ESRD cases with different iPTH levels. RORyt and STAT3 levels indicated a significant increase in high iPTH (> 300) compared to the normal (> 150 and 300 <) (p=0.0262), (p=0,0017) and low patients (150 >) (p<0.0001), (p<0.0001) respectively. β actin was used for the normalization of mRNA. The data are expressed as mean ± SD. P< 0.05 was considered statistically significant (RORγt: receptor-related orphan nuclear receptor γt; STAT3: signal transducer and activator of transcription 3; Th17: T-helper type 17; PTH: Parathyroid hormone; ESRD: End-stage renal disease)

Supplementary Fig. 2. Western blot analysis of STAT3 protein in isolated T cells of patients with ESRD with different iPTH levels. STAT3 levels indicated a significant increase in high iPTH (> 300) in comparison to the normal PTH ((> 150 and 300 <) (p=0.0211) and hypoparathyroidism patients (150 >) (p=0.0255). Results are expressed as mean ± SD. P < 0.05 was considered statistically significant (STAT3: signal transducer and activator of transcription 3; PTH: Parathyroid hormone; ESRD: End-stage renal disease).

Supplementary Fig. 3. Th17 cell-associated cytokines evaluation in the supernatant of isolated T cells. The concentrations of IL-17 and IL-23 were remarkably enhanced in high iPTH cases (> 300) in comparison to the control ((> 150 and 300 <) (p=0.0346), (p=0,232) and with low iPTH patients (150 >) (p=0.0059), (0.0087), respectively. Data are expressed as mean ± SD. P < 0.05 was considered statistically significant (IL: interleukin, Parathyroid hormone, ESRD: End-stage renal disease).