

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

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2 **Evaluation of T helper17 as skeletal homeostasis factor in peripheral blood**
3 **mononuclear cells and T helper cells of end-stage renal disease cases with impaired**
4 **parathyroid hormone**
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6 ***Running title: T helper17 cells in end stage renal diseases with impaired parathyroid***
7 ***hormone***

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36 **Abstract**

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

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

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

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

54

55 **Keywords:** Chronic kidney disease; IPTH; Th17; STAT3; ROR γ t

56 **1. Introduction**

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

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

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

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

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77

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 μ m).

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

106 ***2.3. Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) for relative gene*** 107 ***expression of STAT3 and ROR- γ t***

108 Total RNA extraction was performed using an RNA extraction kit (SinaClon, Tehran, Iran)
109 according to the manufacturer's instructions. The isolated RNA was quantified by
110 spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc., Wilmington, USA). The c-
111 DNA was first synthesized with a c-DNA synthesis kit (Thermo Fisher, Waltham, MA) in 20 μ l
112 reaction volume with Eppendorf Mastercycle EP Gradient thermal cycler. Next, it was subjected
113 to RT-qPCR (Rotorgene, Qiagen, Germany) by applying master-mix SYBR green (Ampliqon A/S,
114 Denmark) and the primer sets of each target gene as follows: *STAT3* forward
115 5'CTTTGAGACCGAGGTGTATCACCC3' and reverse 5'GGTCAGCATGTTGTACCACAGG3'; *ROR-*
116 *γ t* forward 5'GAGGAAGTGACTGGCTACCAGA3' and reverse
117 5'GCACAATCTGGTCATTCTGGCAG3'. Moreover, 5'CACCATTGGCAATGAGCGGTTC3' forward
118 and 5'AGGTCTTTGCGGATGTCCACGT3' reverse were used for *β -actin* as the housekeeping gene
119 (9). Relative gene expression was evaluated by the $2^{-\Delta\Delta ct}$ method.

120 ***2.4. Detecting IL-17 and IL-23 levels by enzyme-linked immunosorbent assay (ELISA)***

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

124 **2.5. STAT3 Western Blotting assay**

125 Total protein was extracted from lysed PBMCs, and T cells were isolated. The protein
126 concentration of each sample was measured by the BCA assay kit (Merck, Germany). An equal
127 amount of protein was separated from all samples by the SDS-PAGE method. Afterward, samples
128 were electro-transferred to nitrocellulose membranes. For blocking, 5% skimmed milk solution
129 (1X TBST) was applied for 1 hour at room temperature (RT), followed by overnight incubation at
130 4°C. Mouse anti-human STAT-3 (0.1 µg/mL, R&D systems) were used as specific primary
131 antibodies. The next day, membranes were washed three times with 1xTBST and then underwent
132 1 h of incubation with a secondary antibody at RT. After that, membranes were washed and used
133 for visualization (10).

134 **2.6. Magnetic-activated cell sorting (MACS)**

135 CD3 + T cells were isolated using the MACS technique with a negative selection protocol
136 (Miltenyi Biotec, San Diego). Phosphate-buffered saline (PBS) (Sigma-Aldrich, Germany) was
137 used for rinsing isolated cells. Next, 5 mL of supplemented medium with heat-inactivated fetal
138 calf serum 10 % (FBS), penicillin (100 U/mL), L-glutamine (200 mM), and phorbol myristate
139 acetate (10 ng/mL) (PMA; eBioscience, San Diego, CA) were used to cultivate CD3 + T cells and
140 incubate them for 48 h at 37°C and 5% CO₂. The cell culture supernatant was evaluated for
141 detecting cytokine levels in CD3 + T cells by enzyme-linked immunosorbent assay (ELISA). Also,
142 real-time polymerase chain reaction (RT-PCR) and western blotting analysis were done on
143 separated cells.

144 **2.7. Statistical analysis**

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

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

149 **2. 3. Results**

150 ***3.1. General aspects of the study population***

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

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

161 ***3.2. The frequency of circulating Th17 cells in the peripheral blood of the studied group***

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

166 ***3.3. The mRNA expression level of master Th17-related transcription factors***

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

172 0.0387) and also with the low iPTH group in STAT3 ($P = 0.0012$) and ROR γ t ($P = 0.0001$) in
173 PBMC. In addition, these results were confirmed by evaluating the factors mentioned in the T cells
174 population, presented in Supplementary Fig. 1 and Table 1.

175 ***3.4. Detection of Th17-related transcription protein level***

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

182 ***3.5. Levels of Th17-related cytokines in studied cases***

183 The relation between the PTH levels and Th17-associated cytokines was identified by evaluating
184 the supernatant of cultured PBMCs and T cells population (Supplementary Fig. 3) by ELISA. As
185 indicated in Fig. 4 and Table 1, higher IL-17 and Il-23 levels were detected in HD patients with
186 high iPTH compared to normal ($P = 0.346$; $P = 0.0232$) and low iPTH groups ($P = 0.0059$; $P =$
187 0.0087), respectively. Also, evaluation the supernatant of the cultured T cells population verified
188 these results (Supplementary Fig. 3 and Table 2). Finally, no considerable variation was detected
189 in the mentioned cytokines between the low iPTH and normal iPTH groups in HD patients.

190

191 4. Discussion

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

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

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

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

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

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

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

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

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

249

250 **Conclusion**

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

257

258 **Declaration of Competing Interest**

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

261

262

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363 **Table 1:** Demographical and immunological features of hemodialysis patients with different
 364 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

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

368

369 **Figure legends**

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

377

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

385

386 **Fig. 3. Western blot analysis of STAT3 protein in PBMCs of patients with ESRD with**
387 **different iPTH levels.** STAT3 levels indicated a significant increase in high iPTH (> 300) in
388 comparison to the normal PTH ((> 150 and 300 <) (P = 0.0010) and hypoparathyroidism patients
389 (150 >) (P = 0.0053). Results are expressed as mean ± SD. P < 0.05 was considered statistically
390 significant (PBMCs: peripheral blood mononuclear cells; STAT3: signal transducer and activator
391 of transcription 3; PTH: Parathyroid hormone; ESRD: End-stage renal disease).

392

393 **Fig. 4. Th17 cell-associated cytokines evaluation in the supernatant of cultured.** The
394 concentrations of IL-17 and IL-23 were remarkably enhanced in high iPTH cases (> 300) in
395 comparison to the control ($(> 150$ and $300 <)$ ($P = 0.0346$; $P = 0.232$) and with low iPTH patients
396 ($150 >$) ($P = 0.0059$; $P = 0.0087$), respectively. Data are expressed as mean \pm SD. $P < 0.05$ was
397 considered statistically significant (IL: interleukin, PBMCs: peripheral blood mononuclear cells,
398 Parathyroid hormone, ESRD: End-stage renal disease).

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412 **Supplementary Table_1:** Isolated T cell characteristics of hemodialysis patients with different
 413 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

414 <150: Hemodialysis patients with under 150 IPTH; 150<>300: Hemodialysis patients with
 415 between 150 and 300 IPTH; >300: Hemodialysis patients with more than 300 IPTH. p< 0.05 was
 416 considered as significant.

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

436
437 **Supplementary Fig. 2. Western blot analysis of STAT3 protein in isolated T cells of patients**
438 **with ESRD with different iPTH levels.** STAT3 levels indicated a significant increase in high
439 iPTH (> 300) in comparison to the normal PTH ((> 150 and 300 <) (p=0.0211) and
440 hypoparathyroidism patients (150 >) (p=0.0255). Results are expressed as mean \pm SD. P < 0.05
441 was considered statistically significant (STAT3: signal transducer and activator of transcription 3;
442 PTH: Parathyroid hormone; ESRD: End-stage renal disease).

443
444 **Supplementary Fig. 3. Th17 cell-associated cytokines evaluation in the supernatant of**
445 **isolated T cells.** The concentrations of IL-17 and IL-23 were remarkably enhanced in high iPTH
446 cases (> 300) in comparison to the control ((> 150 and 300 <) (p=0.0346), (p=0,232) and with low
447 iPTH patients (150 >) (p=0.0059), (0.0087), respectively. Data are expressed as mean \pm SD. P <
448 0.05 was considered statistically significant (IL: interleukin, Parathyroid hormone, ESRD: End-
449 stage renal disease).

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