Association of plasma *ortho*-tyrosine/*para*-tyrosine ratio with responsiveness of erythropoiesis-stimulating agent in dialyzed patients

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Objectives: Patients with end-stage renal failure (ESRF) treated with erythropoiesis-stimulating agents (ESAs) are often ESA-hyporesponsive associated with free radical production. Hydroxyl free radical converts phenylalanine into *ortho*-tyrosine, while physiological isomer *para*-tyrosine is formed enzymatically, mainly in the kidney. Production of '*para*-tyrosine' is decreased in ESRF and it can be replaced by *ortho*-tyrosine in proteins. Our aim was to study the role of tyrosines in ESA-responsiveness.

Methods: Four groups of volunteers were involved in our cross-sectional study: healthy volunteers (CONTR; $n = 16$), patients on hemodialysis *without* ESA-treatment (non-ESA-HD; $n = 8$), hemodialyzed patients *with* ESA-treatment (ESA-HD; $n = 40$), and patients on continuous peritoneal dialysis (CAPD; $n = 21$). Plasma *ortho*-, *para*-tyrosine, and phenylalanine levels were detected using a high performance liquid chromatography (HPLC)-method. ESA-demand was expressed by ESA-dose, ESA-dose/body weight, and erythropoietin resistance index₁ (ERI₁, weekly ESA-dose/body weight/hemoglobin).

Results: We found significantly lower *para*-tyrosine levels in all groups of dialyzed patients when compared with control subjects, while in contrast *ortho*-tyrosine levels and *ortho*-tyrosine/*para*-tyrosine ratio were comparatively significantly higher in dialyzed patients. Among groups of dialyzed patients the *ortho*-tyrosine level and *ortho*-tyrosine/*para*-tyrosine ratio were significantly higher in ESA-HD than in the non-ESA-HD and CAPD groups. There was a correlation between weekly ESA-dose/body weight, ERI₁, and *ortho*-tyrosine/*para*-tyrosine ratio ($r = 0.441$, $P = 0.001$; $r = 0.434$, $P = 0.001$, respectively). Our most important finding was that the *ortho*-tyrosine/*para*-tyrosine ratio proved to be an independent predictor of ERI₁ ($\beta = 0.330$, $P = 0.016$). In these multivariate regression models most of the known predictors of ESA-hyporesponsiveness were included.

Discussion: Our findings may suggest that elevation of the ratio of *ortho*-tyrosine/*para*-tyrosine could be responsible for decreased ESA-responsiveness in dialyzed patients.

Keywords: Darbepoetin alfa, Kidney failure, Chronic, Oxidative stress, Hydroxyl radical

Introduction

Anemia is a common complication in patients suffering from chronic kidney disease (CKD). Recombinant human erythropoietin (rHuEPO) has been used in CKD since 1989, resulting in the improvement of quality of life. Approximately 10% of the patients on rHuEPO therapy are hyporesponsive, associated with an increased risk of death.¹ Any consensus definition is lacking but according to the review by Bamgbolaan evaluation of erythropoiesis-stimulating agent (ESA)-resistance is suggested when

the hemoglobin deficit is persistent despite more than 3 months' treatment with ≥ 400 IU/kg or $\geq 20\,000$ IU/week of rHuEPO.² The European Best Practice Guidelines (EBPG) defines ESA-resistance as an epoetin dose > 300 IU/kg/week.³ Kidney Disease Improving Global Outcomes (KDIGO) classifies patients as having initial ESA-hyporesponsiveness if they have no increase in Hb concentration from baseline after the first month of ESA treatment on appropriate weight-based dosing, while subsequent (acquired) ESA-hyporesponsiveness is defined as being when patients after treatment with stable doses of ESA require two increases in ESA doses up to 50% beyond the dose at which they had been stable in an effort to maintain a stable Hb concentration.⁴

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The reasons for ESA-hyporesponsiveness are not yet clearly identified. The roles of iron, folate, cobalamin or carnitine deficiency, bleeding, inflammation, angiotensin converting enzyme (ACE)-inhibitor administration, uremic toxins, insufficient dialysis, hyperparathyroidism, and malignancy are suggested by several studies.^{3,5,6} Van der Putten *et al.* have highlighted the role of cytokine-inducible sulfhydryl group containing protein (CIS), which is upregulated by inflammatory cytokines and which binds to the EPO-receptor (EPO-R) and inhibits EPO-dependent cell proliferation. They also evaluated the influence of hematopoietic cell phosphatase in the inhibition of signal transduction of EPO-R.¹ In the *post hoc* study of Rossert *et al.*⁶ diabetes mellitus was the primary cause of kidney disease among the non-responder group. These findings are consistent with the observations of Abe *et al.*, who state that insulin resistance is associated with ESA-hyporesponsiveness since the ESA-dose was significantly higher in the group of diabetic patients. According to their investigations, homeostasis model assessment-insulin resistance (HOMA-IR), hemoglobin, IL-6, TNF- α , and high molecular weight adiponectin were the independent predictors of the required ESA-dose.⁷

Several publications have proven that oxidative stress plays a major role in hormone resistances, such as insulin resistance.^{8,9} Bearing the above-mentioned observations in mind we assumed that oxidative stress could be a common mediator in the development of insulin resistance as well as ESA-resistance. Oxidative stress is defined as the loss of balance between pro- and antioxidant systems. This imbalance results an overproduction of such free radicals as hydroxyl radical (HO $\cdot$) leading to direct tissue damage or functional impairment. Hydroxyl radical converts l-phenylalanine into *ortho*-tyrosine, an isomer of the natural amino acid *para*-tyrosine.^{10,11} Consequently, elevated levels of *ortho*-tyrosine detect hydroxyl radical-induced tissue damage. On the other hand, *para*-tyrosine is formed enzymatically under physiological circumstances in the liver and – to a greater proportion – in the kidney.

It is well known that high levels of oxidative stress are present in patients with end-stage renal failure (ESRF).^{12–14} There are data proving that inflammation and duration of dialysis treatment are the most important factors in the development of oxidative stress in these patients.¹⁴ In our previous studies we have shown that levels of oxidatively modified derivatives of phenylalanine (such as *ortho*-tyrosine) were significantly elevated among such circumstances when oxidative stress develops in the human body. A higher *ortho*-tyrosine concentration was measured in cataractous lenses in comparison with that of the control group.¹⁵ In another study of

our workgroup, plasma and urine levels of *para*- and *ortho*-tyrosine were measured in patients with chronic kidney disease (CKD) and/or diabetes (DIAB-CKD/DIAB). Urinary *ortho*-tyrosine excretion was higher in all groups of patients than in the control group, and higher in DIAB patients and DIAB-CKD patients than in CKD patients.¹⁶ Formerly we had also observed that the total urinary albumin/creatinine ratio and urinary non-immunoreactive albumin/creatinine ratio show a significant correlation with the urinary *ortho*-tyrosine/creatinine ratio in patients with acute ischemic stroke.¹⁷ Finally, administration of natural antioxidant resveratrol significantly decreased *ortho*-tyrosine excretion and improved insulin resistance in patients with type 2 diabetes mellitus.¹⁸ Vivekanadan-Giri *et al.*¹⁹ found the *ortho*-tyrosine level to be elevated in the aortic proteins of diabetic monkeys.

It is known that structural analogs of *para*-tyrosine, such as 3-iodotyrosine, 3-fluorotyrosine, and 3,4-dihydroxy-l-phenylalanine, are incorporated by post-transcriptional mechanisms into structural or functional proteins of tissues.²⁰ Gurer-Orhan *et al.*²¹ have shown that the cytotoxicity of another hydroxyl free radical product of phenylalanine, *meta*-tyrosine, may be mediated by the concentration-dependent integration of this abnormal amino acid into cellular proteins. Furthermore, both *meta*-tyrosine and *ortho*-tyrosine inhibited tumor cell proliferation in different murine models of cancer. It has been shown that the antitumor effects of these two pathological tyrosine isoforms were mediated, in part, by early inhibition of the mitogen-activated protein/extracellular signal-regulated kinase (MAP/ERK) pathway and inactivation of STAT3.²²

Intracellular signal transduction of the EPO-receptor (EPO-R) is well described, but complex.^{23,24} The receptor is activated on eight cytoplasmic tyrosine domains, and continued in the downstream signal of the JAK2/STAT1–5 pathway.²³ EPO also stimulates the catalytic activity of several mitogen-activated protein kinases such as extracellular-regulated kinases 1/2 (Erk1/2).²⁴ Tyrosine phosphorylation plays an essential role in both of the above-mentioned signaling pathways.^{23–25} Moreover, EPO-R activation may cause hydroxyl free radical production through stimulation of the NADPH-oxidase enzyme.²⁶ We hypothesized that altered availability of *para*- and *ortho*-tyrosine may be – in part – the cause and mediator of decreased ESA-responsiveness observed in patients on dialysis.

Materials and methods

Subjects

Four groups of volunteers were involved in our cross-sectional study. We selected 69 patients from our

dialysis unit (University of Pécs 2nd Department of Medicine and Nephrology Center, Fresenius Medical Care) and 16 healthy volunteers (CONTR, median age 40 years, eight males/eight females, data not shown in the table). Patients with acute infections, malignancy, and active autoimmune disease were excluded from the study. Forty-eight patients were treated with hemodialysis (HD), while 21 with continuous peritoneal dialysis (CAPD). The group was included in the cross-sectional study because they were known to respond well to ESA and have better survival. Out of the hemodialyzed patients, 8 did not receive ESA (non-ESA-HD), while 40 were treated with ESA (ESA-HD). They were administered intravenous darbepoietin- α (DA) weekly or twice a month. Patients on CAPD were receiving subcutaneous DA. Hemodialyzed patients had been undergoing regular HD three times a week for 4 hours. In all cases we used an FX 50 or 60 Cor Diax high-flux dialyser with Helixone® membrane (Fresenius Medical Care), and a blood flow rate of 180–400 ml/min. The study was approved by the Ethical Committee of the Medical Faculty of the University of Pécs. All patients had given their informed consent.

Routine analytical procedures

Hemoglobin, hematocrit, red blood cell count, serum sodium, potassium, calcium, phosphate, parathyroid hormone (PTH), iron, transferrin, transferrin saturation, ferritin, creatinine, blood urea nitrogen, high sensitivity C-reactive protein (hsCRP), total protein, albumin, total cholesterol, HDL-cholesterol, and triglyceride levels were determined by standard laboratory techniques. All of the blood samples were drawn just before the actual HD-treatment. ESA-demand was expressed using different ESA indices such as $ERI_1 = \text{ESA-dose/body weight/hemoglobin}$; $ERI_2 = \text{ESA-dose/body weight/hematocrit}$; $ERI_3 = \text{ESA-dose/body weight/red blood cell count}$.

High performance liquid chromatography (HPLC) analysis

Blood samples were obtained by way of vein puncture, in tubes containing EDTA. Plasma was obtained by centrifugation and was stored at -80°C pending further examination. Thereafter 125 μl trichloroacetic acid (TCA) was added to 500 μl plasma and then samples were incubated on ice for 30 minutes. Subsequently precipitate was separated by centrifugation. The supernatant was filtered by a syringe filter (0.2 μm) before analysis. Finally plasma *ortho*-, *para*-tyrosine, and phenylalanine levels were determined using reverse phase-HPLC (C_{18} silica column, $250 \times 4 \text{ mm}$) with fluorescence detection ($\lambda_{\text{EX}} = 275 \text{ nm}$; $\lambda_{\text{EM}} = 305 \text{ nm}$ for the tyrosines and $\lambda_{\text{EX}} = 258 \text{ nm}$; $\lambda_{\text{EM}} = 288 \text{ nm}$ for phenylalanine) as

described earlier.¹⁶ Concentrations were calculated using an external standard.

Investigating ESA-effect on *ortho*-tyrosine level

Five patients of the ESA-HD group were included in a longitudinal analysis that consisted of two sessions. The first session was a HD *without* DA-treatment (ESA–), while the second session of the same patients was a HD *with* DA-treatment (ESA+; mean 28 μg of DA) at time point ‘0’. Blood samples were obtained five times (60 minutes before the end of HD (–60), at the end of HD (0) and 60, 120 and 180 minutes after the end of HD). The time kinetics of plasma *ortho*-tyrosine levels at both sessions was examined.

Statistical analysis

Central tendency and width of distribution were expressed using mean $\pm$ SD in the cases of normally and, using median(interquartile range), in case of non-normally distributed data. Kolmogorov–Smirnov test was used to test normality. Differences between groups were analyzed using ANOVA with Bonferroni *post hoc* test (normal distribution) or using Kruskal–Wallis and Mann–Whitney *U* tests (non-normal distribution). χ^2 test was used to compare groups in the case of categorical variables. Correlations were calculated using the Spearman correlation. Linear regression analysis was performed using a stepwise method. *P* values <0.05 were considered to be statistically significant. All statistical analyses were performed with SPSS 20 software (IBM).

Results

Patient characteristics

Baseline characteristics of the dialyzed patients can be seen in Table 1. Among the hemodialyzed patients, diabetic nephropathy (36%) was the leading cause of ESRF. Other causes were benign nephrosclerosis (19%), polycystic kidney disease (13%), primary glomerulopathy (10%), chronic pyelonephritis (10%), and chronic tubulointerstitial nephritis (6%). In three cases (6%) the cause of ESRF remained unknown. Among patients with CAPD, polycystic kidney disease (29%) was most commonly found to be behind ESRF. Further causes were nephrosclerosis (24%), diabetic nephropathy (19%), primary glomerulopathy (14%), and chronic pyelonephritis (9%). In one case (5%) contrast media nephropathy led to ESRF.

Levels of plasma *para*-tyrosine

Plasma *para*-tyrosine levels were significantly lower in all groups of dialyzed patients than in the control subjects (non-ESA-HD, $35.10(17.24) \mu\text{M}$, $P = 0.001$; ESA-HD, $36.25(13.67) \mu\text{M}$, $P < 0.001$; CAPD, $39.53(13.20) \mu\text{M}$, $P < 0.001$; CONTR, $57.24(18.19) \mu\text{M}$),

Table 1 Clinical parameters of dialysis patients

Group	Non-ESA-HD	ESA-HD	CAPD
<i>n</i>	8	40	21
Age (years)	72 (22)	77 (16)	58 (25) ^{a,b}
Male/female	5/3	12/28	12/9
Dialysis duration (months)	40 (43)	34 (63)	8 (10) ^{b,c}
Restdiuresis (ml)	200 (550)	200 (575)	1500 (1300) ^{b,c}
<i>Kt/V_{urea}</i>	1.72 (0.61)	1.67 (0.33)	2.00 (0.71) ^{a,b}
ESA-dose (μg/week)	–	15 (15)	5 (20) ^d
ESA-dose/body weight (μg/kg/week)	–	0.2500 (0.2604)	0.0405 (0.2487) ^b
ERI ₁ (μg/kg/week/g/l)	–	0.0023 (0.0025)	0.0003 (0.0026) ^b
ERI ₂ (μg/kg/week/%)	–	0.0073 (0.0076)	0.0011 (0.0078) ^b
ERI ₃ (μg/kg/week/T/l)	–	0.0630(0.0642)	0.0101 (0.0734) ^b
Hemoglobin (g/l)	122 (21)	113 (11)	116 (16)
Hematocrit (%)	35.4 ± 4.3	33.7 ± 2.9	35.3 ± 4.2
RBC (T/l)	3.84 ± 0.52	3.59 ± 0.38	3.80 ± 0.59
MCV (fl)	92.4 ± 5.2	94.2 ± 5.8	93.5 ± 7.7
MCH (pg)	30.74 (3.23)	31.53 (2.85)	30.40 (4.49)
MCHC (g/l)	334 (30)	330 (13)	327 (14)
Body weight (kg)	72.8 (26.6)	64.3 (17.5)	80.0 (45.8) ^b
Height (m)	1.60 (0.15)	1.63 (0.10)	1.70 (0.13) ^b
BMI (kg/m ²)	28.40 (8.24)	25.24 (5.92)	26.73 (13.21)
Se-Na ⁺ (mmol/l)	140 (8)	140 (5)	141 (4)
Se-K ⁺ (mmol/l)	5.00 (2.10)	4.80 (0.97)	4.30 (0.55) ^{b,c}
Se-Ca ²⁺ (mmol/l)	2.04 ± 0.28	2.17 ± 0.15	2.26 ± 0.25 ^a
Se-PO ₄ ³⁻ (mmol/l)	1.52 (0.92)	1.43 (0.54)	1.71 (0.56)
PTH (pmol/l)	43.79 (46.25)	32.15 (31.51)	25.93 (36.20)
Se-iron (μmol/l)	11.8 (4.4)	10.3 (6.1)	12.1 (4.6)
Se-transferrin (g/l)	1.5 ± 0.3	1.6 ± 0.3	2.0 ± 0.3 ^{b,c}
Transferrin saturation (%)	33.38 (14.93)	26.38 (21.18)	23.04 (10.68) ^c
Se-ferritin (μg/l)	999 ± 331	1056 ± 494	392 ± 246 ^{b,c}
Se-creatinine (μmol/l)	570 (289)	522 (228)	468 (291)
hsCRP (mg/l)	10.5 (10.3)	9.7 (9.8)	6.2 (6.1)
Se-total protein (g/l)	63.5 ± 7.3	64.2 ± 5.2	65.0 ± 7.1
Se-albumin (g/l)	36.1 ± 4.6	38.4 ± 3.2	39.5 ± 5.0
Se-total cholesterol (mmol/l)	4.35 (0.61)	4.64 (1.42)	5.03 (1.33) ^a
Se-HDL-cholesterol (mmol/l)	0.96 (0.32)	1.12 (0.52)	0.94 (0.46) ^d
Calculated se-LDL-cholesterol (mmol/l)	2.51 (0.61)	2.81 (1.07)	3.00 (1.20)
Se-non-HDL-cholesterol (mmol/l)	3.30 (0.88)	3.50 (1.11)	4.10 (1.42) ^{a,d}
Se-triglyceride (mmol/l)	1.79 (1.36)	1.38 (0.79)	2.46 (2.24) ^b
Systolic blood pressure (mmHg)	125 (10)	120 (20)	120 (30)
Diastolic blood pressure (mmHg)	70 (16)	70 (10)	70 (13)
No. of antihypertensive drugs	3 (2)	3 (3)	3 (4) ^{a,b}
RAAS-inhibitors (%)	50	55	90 ^{a,b}
Diabetes (%)	63	48	43
CVD (%)	75	83	71
Cerebrovascular disease (%)	38	33	14
PAD (%)	50	33	10 ^a

RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; BMI, body mass index; BSA, body surface area; se-, serum; PTH, parathormone; hsCRP, high sensitivity C-reactive protein; RAAS, renin angiotensin aldosterone system; CVD, cardiovascular disease; PAD, peripheral artery disease. ERI₁, ESA-dose/body weight/Hb; ERI₂, ESA-dose/body weight/Htc; ERI₃, ESA-dose/body weight/RBC.

^a*P* < 0.05, as compared to group non-ESA-HD. ^b*P* < 0.01, as compared to group ESA-HD. ^c*P* < 0.01, as compared to group non-ESA-HD. ^d*P* < 0.05, as compared to group ESA-HD. Data represent the median (interquartile range) or mean ± SD.

but there was no difference among non-ESA-HD, ESA-HD, and CAPD groups (Fig. 1A).

Levels of plasma *ortho*-tyrosine and *ortho*-tyrosine/*para*-tyrosine ratio

We detected significantly higher plasma *ortho*-tyrosine levels in groups of dialyzed patients (non-ESA-HD, 162.97(191.24) nM, ESA-HD, 489.92(726.85) nM, CAPD, 54.19(262.91) nM) than in the control subjects (CONTR, 9.74(9.93) nM, all *P* < 0.001). Plasma *ortho*-tyrosine levels were significantly lower in non-ESA-HD and CAPD groups than in the ESA-HD group (*P* = 0.014 and 0.001, respectively),

while there was no significant difference between non-ESA-HD and CAPD patients (Fig. 1B).

Using the plasma *ortho*-tyrosine/*para*-tyrosine ratio, we found the same pattern as in case of plasma *ortho*-tyrosine, i.e. significantly higher plasma *ortho*-tyrosine/*para*-tyrosine ratio was detected in dialyzed patients (non-ESA-HD, 5.1302(5.2187) nM/μM, ESA-HD, 12.6595(24.2529) nM/μM, CAPD, 1.2364(6.1893) nM/μM) than in the control subjects (CONTR, 0.1684(0.1820) nM/μM, all *P* < 0.001). Also plasma *ortho*-tyrosine/*para*-tyrosine ratios were significantly lower in non-ESA-HD and CAPD groups than in the ESA-HD group (*P* = 0.018 and

Figure 1 Level of plasma *para*-tyrosine (A) and *ortho*-tyrosine (B) in controls (CONTR), hemodialyzed patients *without* ESA-treatment (non-ESA-HD), hemodialyzed patients *with* ESA-treatment (ESA-HD), and patients with peritoneal dialysis (CAPD). *para*-Tyr, *para*-tyrosine; *ortho*-Tyr, *ortho*-tyrosine.

$P < 0.001$, respectively), while we found no significant difference between non-ESA-HD and CAPD patients (data not shown).

Association between ESA-demand and plasma *ortho*-tyrosine level or *ortho*-tyrosine/*para*-tyrosine ratio

There was a significant positive correlation between weekly ESA-dose/body weight and plasma *ortho*-tyrosine level ($r = 0.411$; $P = 0.002$) (Fig. 2A) and a somewhat stronger correlation between weekly ESA-dose/body weight and plasma *ortho*-tyrosine/*para*-tyrosine ratio ($r = 0.441$; $P = 0.001$) (Fig. 2B). Moreover, ERI_1 proved to be in strong correlation with both the plasma *ortho*-tyrosine level ($r = 0.400$; $P = 0.002$) (Fig. 2C) and the plasma *ortho*-tyrosine/*para*-tyrosine ratio ($r = 0.434$; $P = 0.001$) (Fig. 2D). As the distribution of *ortho*-tyrosine level and *ortho*-tyrosine/*para*-tyrosine ratio was not normal, these variables had been converted to natural logarithm.

We examined the predictors of ESA-demand in a multivariate regression analysis. ESA-demand was expressed in terms of ESA-dose, ESA-dose/body weight, and different ESA-indices such as ESA-dose/body weight/hemoglobin, ESA-dose/body weight/hematocrit or ESA-dose/body weight/red blood cell count (ERI_1 ; ERI_2 ; ERI_3 , respectively) in different models. We calculated plasma *para*-tyrosine/phenylalanine, plasma *ortho*-tyrosine/phenylalanine, and plasma *ortho*-tyrosine/*para*-tyrosine ratios. These three different tyrosine values were included separately in models, together with already known predictors of ESA-demand. In the first model serum calcium ($se-Ca^{2+}$), serum phosphate ($se-PO_4^{3-}$), parathormone (PTH), restdiuresis, and serum ferritin; while in the second model age, dialysis duration, high sensitivity C-reactive protein (hsCRP), restdiuresis, and serum ferritin were included besides the actual tyrosine value. As a result neither *para*-

tyrosine/phenylalanine nor *ortho*-tyrosine/phenylalanine ratio proved to be an independent predictor in these two models. In almost all cases restdiuresis was the independent predictor (Tables 2 and 3). In contrast, when the *ortho*-tyrosine/*para*-tyrosine ratio was included into the model, it proved to be an independent predictor of the ESA-dose and ESA-indices in both models (Table 4). Finally, we set a third model where kt/v was also included among age, PTH, hsCRP, serum ferritin, and *ortho*-tyrosine/*para*-tyrosine ratio. Also in this case *ortho*-tyrosine/*para*-tyrosine ratio proved to be the independent predictor ($\beta = 0.292$; $P = 0.042$) of ESA-dose/body weight (data not shown in the table).

ESA-effect on plasma *ortho*-tyrosine level

A potential explanation for the observed associations shown in the previous part could be a formation of $HO\cdot$ due to ESA-administration. In that regard, larger ESA doses would not be the consequence, but rather the cause of oxidative stress-related amino acid modification. To rule this out, in a longitudinal analysis, time kinetics of plasma *ortho*-tyrosine levels were compared at two hemodialysis sessions, in the absence of ESA-treatment (ESA-), and in the presence of ESA-therapy (ESA+). As a result, no significant difference was found between the two curves (Fig. 3).

Discussion

In this study we have highlighted the importance of *ortho*- and *para*-tyrosine in decreased ESA-responsiveness. Plasma *para*-tyrosine levels were significantly lower, while plasma *ortho*-tyrosine levels and *ortho*-tyrosine/*para*-tyrosine ratio were significantly higher in dialyzed patients compared to controls. Furthermore, plasma *ortho*-tyrosine level and *ortho*-tyrosine/*para*-tyrosine ratio were significantly lower in the non-ESA-HD and CAPD groups than in the

Figure 2 Correlations between [A] plasma *ortho*-tyrosine or [B] plasma *ortho*-tyrosine/*para*-tyrosine and ESA-dose/body weight. Correlations between [C] plasma *ortho*-tyrosine or [D] plasma *ortho*-tyrosine/*para*-tyrosine and ERI₁ = ESA-dose/body weight/hemoglobin. *ortho*-Tyr, *ortho*-tyrosine; *para*-Tyr, *para*-tyrosine.

ESA-HD group. The *ortho*-tyrosine level and *ortho*-tyrosine/*para*-tyrosine ratio showed a significant and positive correlation with weekly ESA-dose/body weight and ERI₁. The most important finding is that plasma *ortho*-tyrosine/*para*-tyrosine ratio proved to be an independent predictor of ESA-dose, ESA-

dose/body weight, and ESA-indices calculated in different ways in such models, where most of the known predictors of ESA-hyporesponsiveness were also included. Neither the plasma *ortho*-tyrosine/phenylalanine nor the *para*-tyrosine/phenylalanine ratio were independent predictors of ESA-demand in

Table 2 Predictors of ESA-demand (plasma *para*-tyrosine/phenylalanine ratio included)

Dependent	Model 1			Model 2		
	Independent predictor	β	<i>P</i>	Independent predictor	β	<i>P</i>
ESA-dose	Restdiuresis	-0.256	0.038	Restdiuresis	-0.256	0.038
ESA-dose/body weight	Restdiuresis	-0.329	0.007	Restdiuresis	-0.329	0.007
ERI ₁	Restdiuresis	-0.302	0.014	Restdiuresis	-0.302	0.014
ERI ₂	Restdiuresis	-0.304	0.013	Restdiuresis	-0.304	0.013
ERI ₃	Restdiuresis	-0.299	0.015	Restdiuresis	-0.299	0.015

Model 1: se-Ca²⁺, se-PO₄³⁻, PTH, restdiuresis, se-ferritin, and plasma *para*-tyrosine/phenylalanine.

Model 2: age, dialysis duration, hsCRP, restdiuresis, se-ferritin, and plasma *para*-tyrosine/phenylalanine.

ERI₁, ESA-dose/body weight/hemoglobin; ERI₂, ESA-dose/body weight/hematocrit; ERI₃, ESA-dose/body weight/red blood cell count.

Method: stepwise.

Table 3 Predictors of ESA-demand (plasma *ortho*-tyrosine/phenylalanine ratio included)

Dependent	Model 1			Model 2		
	Independent predictor	β	P	Independent predictor	β	P
ESA dose	—	—	—	—	—	—
ESA-dose/body weight	Restdiuresis	−0.298	0.030	Restdiuresis	−0.298	0.030
ERI ₁	Restdiuresis	−0.275	0.046	Restdiuresis	−0.275	0.046
ERI ₂	Restdiuresis	−0.277	0.045	Restdiuresis	−0.277	0.045
ERI ₃	Restdiuresis	−0.273	0.048	Restdiuresis	−0.273	0.048

Model 1: se-Ca²⁺, se-PO₄^{3−}, PTH, restdiuresis, se-ferritin, and plasma *ortho*-tyrosine/phenylalanine.

Model 2: age, dialysis duration, hsCRP, restdiuresis, se-ferritin, and plasma *ortho*-tyrosine/phenylalanine.

ERI₁, ESA-dose/body weight/hemoglobin; ERI₂, ESA-dose/body weight/hematocrit; ERI₃, ESA-dose/body weight/red blood cell count.

Method: stepwise.

both models, suggesting that neither an elevation of the *ortho*-tyrosine level or a decrease in the *para*-tyrosine level per se is sufficient predictor of the raising ESA-demand, but that combined and inverse alteration of these amino acid isomers is in a strong association with decreased ESA-responsiveness. This may mean that among circumstances when pathological *ortho*-tyrosine concentration is increased and at the same time physiological *para*-tyrosine is lowered (e.g. in ESRF), translational integration of *ortho*-tyrosine instead of *para*-tyrosine into the signaling proteins could change the intracellular outcome of EPO-receptor activation, causing lowered ESA-responsiveness (Fig. 4). To rule out a confounder effect, finally we provided evidence that ESA-treatment neither increases nor suppresses HO₂•-production. In a previous study, lipid peroxidation products as *malondialdehyde* and *4-hydroxynonenal* were not elevated in patients with long-term rHuEPO-treatment, leading the authors to conclude that rHuEPO-treatment per se does not inhibit oxidative stress.²⁷

In another *post hoc* study based on CHOIR trial, higher epoetin-alfa doses were associated with increased risks for cardiovascular events, suggesting that epoetin-alfa could have a cardiovascular toxic effect.²⁸ However, our findings raise the possibility of a different mechanism, since an elevated level of hydroxyl radical-derived products may cause

decreased responsiveness to ESAs, leading to higher ESA-demand, and that this could also be at the background of higher risk for cardiovascular events. Namely, there is evidence that these products can interfere with the MAP/ERK signaling pathway and that direct inhibition of ERK1/2 can lead to exacerbated cardiomyocyte death and impaired cardiac function.^{22,29}

The association between oxidative stress and ESA-hyporesponsiveness has been investigated in several further studies. Serum and erythrocyte *thiobarbituric acid reactive substances* (TBARS) and serum superoxide and hydroxyl radical scavenging activities were measured in hemodialysis patients receiving different doses of rHuEPO. Higher TBARS levels were found in erythrocytes of patients receiving high doses of rHuEPO (9000 U/week) as compared with patients with no ESA-treatment, while no significant difference was found in serum TBARS levels. A diminution of serum hydroxyl radical scavenging activity was observed in subjects with high-dose rHuEPO treatment.³⁰ This finding is in agreement with ours, since such a suppressed hydroxyl free radical scavenging activity could lead to elevated hydroxyl radical levels, resulting in higher levels of oxidative derivatives of phenylalanine (as *ortho*-tyrosine).

In another study the serum level of the hydroxyl free radical-DNA product *8-hydroxy-2'-deoxyguanosine*

Table 4 Predictors of ESA-demand (plasma *ortho*-tyrosine/*para*-tyrosine ratio included)

Dependent	Model 1			Model 2		
	Independent predictor	β	P	Independent predictor	β	P
ESA-dose	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.418	0.002	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.366	0.007
ESA-dose/body weight	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.394	0.004	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.394	0.004
ERI ₁	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.330	0.016	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.330	0.016
ERI ₂	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.343	0.012	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.343	0.012
ERI ₃	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.310	0.024	<i>ortho</i> -Tyr/ <i>para</i> -Tyr	0.310	0.024

Model 1: se-Ca²⁺, se-PO₄^{3−}, PTH, restdiuresis, se-ferritin, and plasma *ortho*-tyrosine/*para*-tyrosine.

Model 2: age, dialysis duration, hsCRP, restdiuresis, se-ferritin, and plasma *ortho*-tyrosine/*para*-tyrosine.

ERI₁, ESA-dose/body weight/hemoglobin; ERI₂, ESA-dose/body weight/hematocrit; ERI₃, ESA-dose/body weight/red blood cell count.

Method: stepwise.

ortho-Tyr, *ortho*-tyrosine; *para*-Tyr, *para*-tyrosine.

Figure 3 Time kinetics of plasma *ortho*-tyrosine level when DA is not administered (ESA-) and when administered (ESA+). *, end of HD, at ESA+ the time of DA administration. Pairwise comparisons of ESA- and ESA+ at each time point were not significant. Data represent mean $\pm$ SD. *ortho*-Tyr, *ortho*-tyrosine.

(8-OHdG) was examined in patients on HD with different weekly dosages of rHuEPO. A significantly higher 8-OHdG level was measured in HD patients when compared with that of healthy control subjects. 8-OHdG was positively correlated with patients' age, but not with dialysis duration. Furthermore, 8-OHdG showed a strong positive correlation with the rHuEPO dose and the ratio of the weekly rHuEPO dose/hemoglobin. All of these data are similar to that found in our study and neither their study nor ours proved any correlation with inflammatory or nutritional markers.³¹

Although there are data proving that ESA-hyporesponsiveness and diabetes commonly appear together and diabetic patients often require a higher ESA-dose, in our study we did not find a significant difference in the frequency of diabetes between non-ESA-HD and ESA-HD groups. Also, no correlation was found between daily insulin doses and ESA-doses or ESA-indices, although these may be due to the relative low number of diabetic cases in our study.

Our findings may suggest that it was not oxidative stress or hydroxyl free radical per se causes that decreased ESA-responsiveness, but that rather the

replacement of *para*-tyrosine with *ortho*-tyrosine (e.g. in signaling proteins) could be responsible for lowered ESA-responsiveness. In this regard *ortho*-tyrosine may be a pathogenetic factor: a marker and not only a marker of ESA-hyporesponsiveness.

There are several limitations in our study. The first limitation is the relatively small number of cases under observation. Further large, multicenter and, if possible international studies are needed for significant evaluation of this association. The second limitation is the partially cross-sectional character of our study. Prospective and/or interventional studies are needed to verify the causative association between higher plasma *ortho*- and lower *para*-tyrosine levels and decreased ESA-responsiveness.

We believe that the evaluation of the association between plasma *ortho*-tyrosine/*para*-tyrosine ratio and ESA-responsiveness may be important since therapeutic interventions decreasing oxidative stress or lowering level of substances produced by oxidative stress (e.g. *ortho*-tyrosine) and/or supplementation of the physiological *para*-tyrosine may improve ESA-responsiveness.

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Disclaimer statements

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None.

Conflict-of-Interest

None.

Ethics Approval

The study was approved by the Ethical Committee of the Medical Faculty of the University of Pécs.

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Figure 4 Possible mechanism of development of ESA-hyporesponsiveness in ESRF patients via inhibition of intracellular signaling pathways. * According to the findings of Ruggiero *et al.*²²

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