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Association of Plasma and mRNA Expression Levels of Ghrelin with Nutritional Status in Egyptian Patients with End-Stage Renal Disease on Regular Hemodialysis

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ABSTRACT

Background: Malnutrition is commonly detected in end-stage renal disease (ESRD) and is a key predictor of their survival. In ESRD patients on hemodialysis protein-energy, malnutrition is significantly observed. The plasma and mRNA expression levels of ghrelin are dysregulated in ESRD.

Objective: The current study aimed to investigate the plasma and mRNA expression levels of ghrelin among Egyptian patients with ESRD on regular hemodialysis and to assess their relations with anthropometric measures and laboratory tests as well as nutritional status.

Patients and methods: A case-control study enrolled 45 healthy control and 50 patients with ESRD on regular hemodialysis. Nutritional assessment was done by subjective global assessment scores (SGA). Patients with ESRD were classified according to their nutritional status into mild, moderate, and severe malnutrition groups. Plasma ghrelin levels were measured using an ELISA and mRNA expression of ghrelin was measured using real-time PCR.

Results: The plasma and mRNA expression levels of ghrelin were significantly higher in ESRD patients with mild and moderate malnutrition compared to the control group. However, the levels of plasma ghrelin were meaningfully lower in ESRD patients with severe malnutrition compared to the control group. plasma and ghrelin mRNA expression levels significantly positively correlated with serum creatinine and BUN and significantly negatively correlated with MAMC, Hb, albumin, transferrin and total iron-binding capacity (TIBC) ($P<0.001$). Serum creatinine transferrin, as well as triceps skin fold, were independently correlated with plasma ghrelin. While serum creatinine transferrin and triceps skin fold, as well as TIBC, were independently correlated with ghrelin mRNA expression levels. **Conclusion:** Ghrelin plasma and relative expression levels were significantly higher in ESRD patients with mild and moderate malnutrition compared to control subjects. While the levels were significantly lower in the severe malnutrition group.

Keywords: ESRD, mRNA expression, Ghrelin hemodialysis, Malnutrition, Subjective global assessment score.

INTRODUCTION

Substantial evidence implicates malnutrition as a major cause of morbidity and mortality for patients with end-stage renal disease (ESRD). It may be assumed that around 20%–50% of patients with ESRD suffer from malnutrition. In addition, 70%–75% of patients have protein-energy wasting [1].

A preponderance of evidence suggests that the mechanism of malnutrition in ESRD could be attributed to loss of taste for food, reduction of food intake, increased catabolism, and decreased anabolism [2]. The molecular and hormonal causes of malnutrition in patients with ESRD could be due to a decrease of insulin, growth hormone (GH), and insulin-like growth factor-1 (IGF-1) [3].

Additionally, the presence of high values of inflammatory cytokines, such as TNF-α, C-reactive protein (CRP), and IL-6. ESRD leads to protein catabolism, muscle wasting and appetite suppression [4-6].Ghrelin is an orexigenic peptide hormone [7] that acts at the GH secretagogue receptor (GHSR) [8] to stimulate appetite, carbohydrate utilization, and GH release [9].

Ghrelin works by transducing signals to hypothalamic regulatory nuclei that control energy homeostasis [10].

According to a randomized, placebo-controlled trial conducted by **Wynne *et al.*** [11] they suggested that ghrelin increases food intake in dialysis patients with mild to moderate malnutrition. Interestingly, **Cheung and Make** [12] observed high levels of ghrelin in relation to fat mass in ESRD patients.There are intriguing reports investigating the levels of ghrelin in patients with ESRD, but the results were conflicting.

Thus, we aimed in the current study to investigate the plasma and mRNA expression levels of ghrelin among Egyptian patients with ESRD on regular hemodialysis and to assess their relations with anthropometric measures, laboratory tests as well as nutritional status.

SUBJECTS AND METHOD

This case-control study enrolled 45 healthy control subjects who were matched regarding age, gender, and ethnic origin to 50 patients with ESRD on maintenance hemodialysis thrice weekly.

All patients with ESRD were subjected to nutritional assessment according to subjective global assessment scores (SGA) [13]. Nutritional assessments including history, dietary assessment, physical examination, and anthropometric measures were done as shown in (Figure 1).

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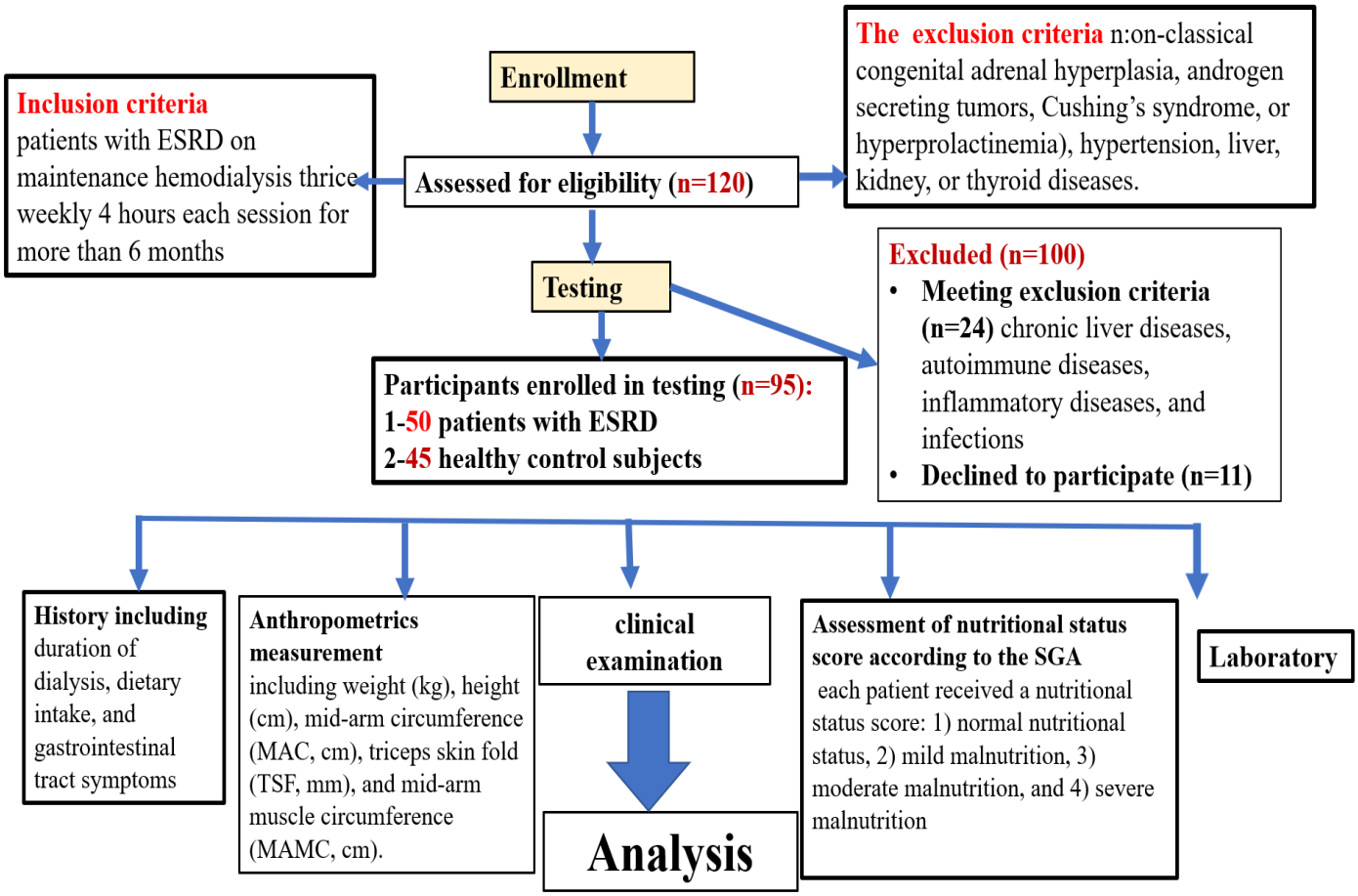

Figure (1): Flow chart of the study.

Laboratory workup

Fasting blood samples was taken. Laboratory investigations of the studied subject were done according to manufacturing protocol in Zagazig University Hospitals. The laboratory investigations included total protein, albumin, blood urea nitrogen (BUN), creatinine, transferrin, serum ferritin, iron, and total iron-binding capacity (TIBC) (were determined by Cobas 6000 & Cobas 8000, Roche diagnostics, Germany). In addition, we investigated CBC by Sysmex Xn 2000.

Detection of ghrelin level by ELISA method

Ghrelin level was measured by a commercially available ELISA kit supplied by DRG® Ghrelin (Human) ELISA International Inc., USA (Catalog No.: EIA-3706).

RNA isolation

The RNA was recovered using a standard reported procedure. The RNA was precipitated with isopropanol, and the pellet was washed with 70% ethanol, air-dried, and dissolved in sterile diethylpyrocarbonate-treated water. The concentration and purity of the RNA were determined using spectrophotometry (Ultrospec® 1100 Pro; Amersham Pharmacia Biotech, Buck, UK) to measure the optical density ratio at 260 and 280 nm.

Real-time RT-PCR of ghrelin

As a template we use from total RNA one microgram to generate cDNA when taking M-MLV reverse transcriptase (Super Bio, Suwon, Korea) and random hexamer priming. Exicycler (Bioneer, Seoul, Korea) used to amplify the cDNA. Real-time PCR analysis was carried out with SYBR® Premix Ex Taq™ (TaKaRa Bio, Tokyo, Japan) and specific primers. The sequences of primers were as follows: Ghrelin: 5'-ATG CTC TGG CTG GAC TTG-3'(sense) and 5'-TCT GCT TGA CCT CCA TCT T-3'(antisense; the gene mRNA levels were normalized using β-actin).

Ethical consent:

An approval of the study was obtained from Zagazig University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

Analysis of data was performed using SPSS v.26. Student's t-tests were used to assess differences between both groups.

Pearson correlation test was used to assess the correlation coefficient between serum and ghrelin mRNA expression levels with anthropometric indices laboratory parameters among ESRD patients.

Linear regression analyses were performed to test the influence of the main independent variables against serum and ghrelin mRNA expression levels (dependent variable) in ESRD patients.

Receiver operating characteristic (ROC) test was done to investigate the diagnostic powers of serum and ghrelin mRNA expression levels among studied subjects. The p-value of ≤ 0.05 was considered statistically significant.

RESULTS

This case-control study enrolled 50 Egyptian patients with ESRD, 49.3 % were females and 51.7% were males, and their mean age was 41.11 ± 15.27 years. In addition, the current study included 45 healthy subjects as control, 50% were females and 50% were males, their mean age was 40.91 ± 13.22 year.

The studied groups were matched for age, sex, BMI, and smoking. As we expected, patients with ESRD had significantly higher values of systolic blood pressure, diastolic blood pressure, serum iron, serum ferritin, serum creatinine, and BUN compared to control group (p <0.001*).

On the other hand, patients with ESRD had significantly lower values of the triceps skin fold, MAMC, Hb, total protein, albumin, transferrin, and TIBC compared to control groups (p <0.001*) **(Table 1)**.

Table (1): Comparison of some measurements in both groups

Variables	Control group (*n* =45)	ESRD patients (*n* =50)	*P* value
Systolic bl. pressure (mmHg)	117.6± 8.86	178.5± 6.63	<0.001*
Diastolic bl. pressure (mmHg)	75.8±4.302	103.8±10.84	<0.001*
Body mass index (kg/m^2)	22.7±1.46	22.19±1.23	0.0512
Triceps skin fold (mm)	6.43±1.21	5.5±1.34	<0.001*
MAMC (cm)	20.6±1.2	18.3±1.1	<0.001*
Hb (g/dl)	13.3±1.2	9.5±1.9	<0.001*
Total protein (g/dl)	6.6±1.1	5.9±1.2	<0.001*
Albumin (g/dl)	4.1±0.46	3.1±0.51	<0.001*
Serum iron (mcg/dl)	64.5±15.73	86.4±20.32	<0.001*
Transferrin (mg/dL)	232.5 ± 17.6	179.5 ±11.3	<0.001*
Serumferritin (ng/Ml)	42.7±5.4	608.5±55.1	<0.001*
TIBC (mcg/dl)	328.6±60.2	181.3±4.2	<0.001*
Serum creatinine (mg/dl)	0.6±0.13	6.43±1.3	<0.001*
BUN (mg/dl)	6.3±1.3	88.3±14.3	<0.001*

MAMC, mid-arm muscle circumference; SGAS, subjective global assessment score.
* *P* < 0.001 when compared obese patients with control group. BUN: blood urea nitrogen; Hb: hemoglobin, TIBC: total iron binding capacity.

Comparison between plasma ghrelin levels (pg/ml) in the studied groups.

Our results showed that there was a highly significant difference between the studied groups regarding plasma ghrelin as the mean value of plasma ghrelin in the control group was 2231.33 ± 446.1. Regards patients with ESRD, for patients with mild malnutrition ghrelin value was 4291.63 ± 870.8, and for patients with moderate malnutrition the value was 4408.52 ± 844.2, while for patients with severe malnutrition the value was 2574.46 ± 1403.9 (P <0.001) (Figure 2a).

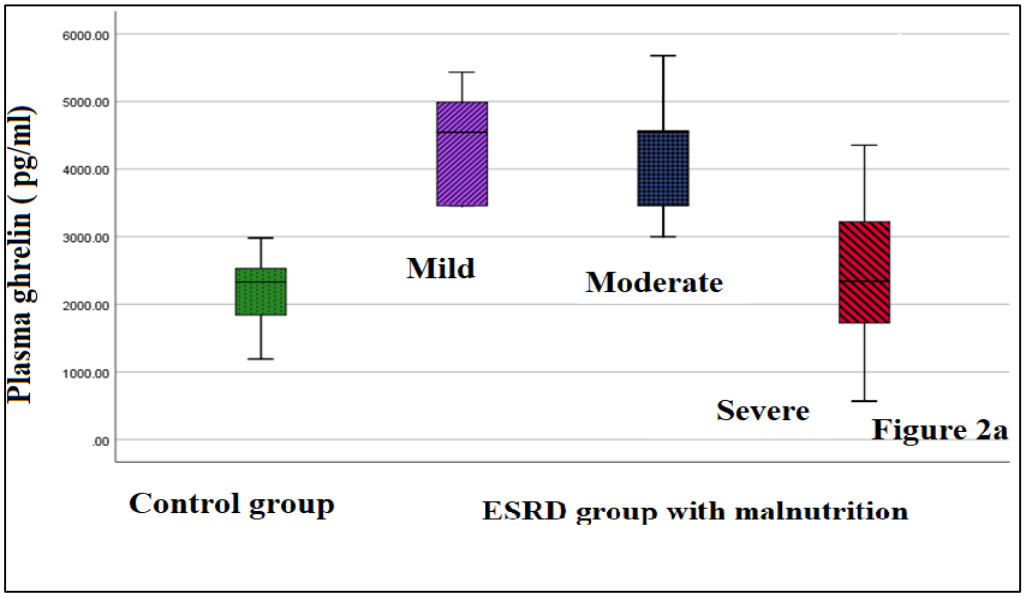

Figure 2a

Figure (2a): Comparison between studied groups as regard plasma ghrelin (pg/ml)

Comparison between ghrelin mRNA expression levels in the studied groups

In an attempt to assess the differences between the studied group regarding ghrelin mRNA expression levels. The current study results showed that there was a highly significant difference between the studied groups regarding ghrelin mRNA expression as the mean value of ghrelin mRNA expression in the control group was 0.85 ± 0.19. Regarding patients with ESRD, for patients with mild malnutrition mean ghrelin value was 1.27 ± 0.65, and for patients with moderate malnutrition the value was 1.45 ± .45, while for patients with severe malnutrition the value was 0.69 ± 0.2586 (P <0.001) (Figure 2b).

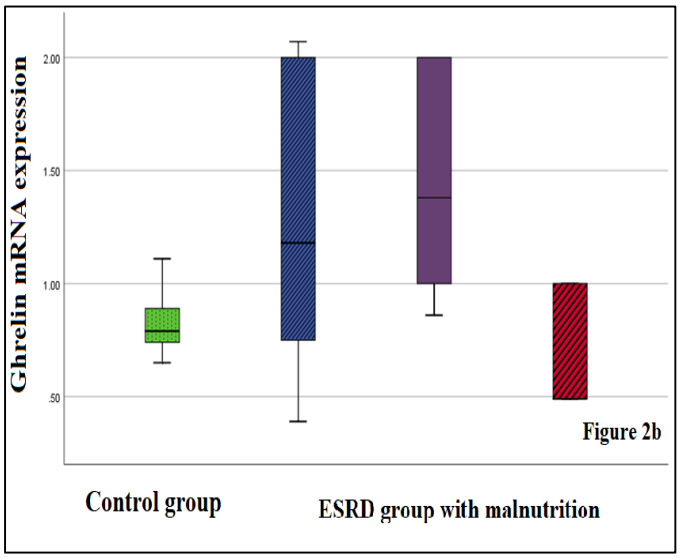

Figure (2b): Comparison between studied groups as regard Ghrelin mRNA expression

Correlations between plasma and ghrelin mRNA expression levels with clinical and laboratory characteristics in patients with ESRD

The current study revealed that plasma and ghrelin mRNA expression levels significantly positively correlated with serum creatinine and BUN and significantly negatively correlated with MAMC, Hb ,albumin, transferrin and TIBC (P < 0.001) (Table 2).

Table (2): Pearson correlation coefficient between plasma and ghrelin mRNA expression levels with anthropometric indices and laboratory parameters in patients with ESRD

Variables		Plasma ghrelin (pg/ml)	Ghrelin mRNA expression
Triceps skin fold	*r*	-0.572	-0.577
	P	<0.001*	<0.001*
MAMC	*r*	-0.408	-0.407
	P	0.064	0.465
Hb	*r*	-0.659	-0.553
	P	<0.001*	<0.001*
Albumin	*r*	-0.447	-0.511
	P	<0.001*	<0.001*
Transferrin	*r*	-0.534	-0.621
	P	<0.001*	<0.001*
TIBC	*r*	-0.433	-0.465
	P	<0.001*	<0.001*
Serum ferritin	*r*	0.086	0.196
	P	0.403	0.056
Serum iron	*r*	0.142	0.167
	P	0.167	0.104
Serum creatinine	r	0.645	0.765
	P	<0.001*	<0.001*
BUN	*r*	0.064	0.465
	P	<0.001*	<0.001*

Linear regression analyses in ESRD patients with mild and moderate malnutrition

For further evaluation of the independent correlations associated with plasma and ghrelin mRNA expression levels in ESRD patients with mild and moderate malnutrition, a linear regression analysis test was done and our results showed that serum creatinine and transferrin, as well as triceps skin fold, were independently correlated with plasma ghrelin. While, serum creatinine, transferrin and triceps skin fold as well as TIBC, were independently correlated with ghrelin mRNA expression levels (P < 0.001) (Table 3).

Table (3): linear regression analyses to test the influence of the main independent variables against plasma and ghrelin mRNA expression levels (dependent variable) in ESRD patients with mild and moderate malnutrition.

Model		Unstandardized Coefficients		Standardized Coefficients	t	P value	95 C.I	
		B	Std. Error	Beta			Lower Bound	Upper Bound
Plasma ghrelin	(Constant)	-66.912	3.670		-0.171	0.865	-845.5	711.70
	Triceps skin fold	21.449	8.078	0.243	2.655	<0.001*	5.389	37.508
	Hb	8.113	36.145	0.179	0.224	0.823	-63.74	79.967
	TIBC	0.232	0.027	0.346	1.834	0.070	-0.019	.484
	S. creatinine	0.045	0.007	0.500	6.755	<0.001*	0.032	.058
	Albumin	-0.084	0.02	-0.592	-1.025	0.308	-0.246	.079
	BUN	1.095	0.037	0.033	0.031	0.975	-68.16	70.349
	Transferrin	-57.902	3.890	-0.571	-4.169	<0.001*	-85.51	-30.29
Ghrelin mRNA expression	(Constant)	-45181.604	17254.639		-2.619	0.010	-45181.6	17254.6
	Albumin	334.839	13.654	0.182	1.823	0.072	334.839	183.654
	Triceps skin fold	-166.703	8.640	-11.947	-3.427	<0.001*	-166.703	48.640
	Hb	-432.687	252.013	-0.178	-1.717	0.090	-432.68	252.013
	BUN	939.690	64.596	1.353	0.974	0.333	939.690	964.596
	S. creatinine	16797.006	915.576	8.728	3.417	<0.001*	16797.1	4915.57
	Transferrin	-916.713	13.446	-0.434	-2.925	<0.001*	-916.71	313.446
	TIBC	-1500.915	55.681	-3.629	-2.802	<0.001*	-1500.9	535.681

The accuracy of ghrelin plasma and relative expression levels for discriminating ESRD patients from the control group by ROC analysis

We investigated the potential diagnostic value of ghrelin plasma and relative expression levels (Figure 3) by the ROC test. When we discriminated patients with ESRD from the control group, the cutoff values were 2340 and 0.850 respectively and the AUC for plasma ghrelin was 0.872 (95% CI =0.792-0.952) and the AUC for ghrelin relative expression levels was 0.697 (95% CI =0.587-0.892). Additionally, the sensitivities and the specificities were 92.2% and 63.2% for plasma ghrelin 92.2% and 64.6% for ghrelin relative expression levels respectively.

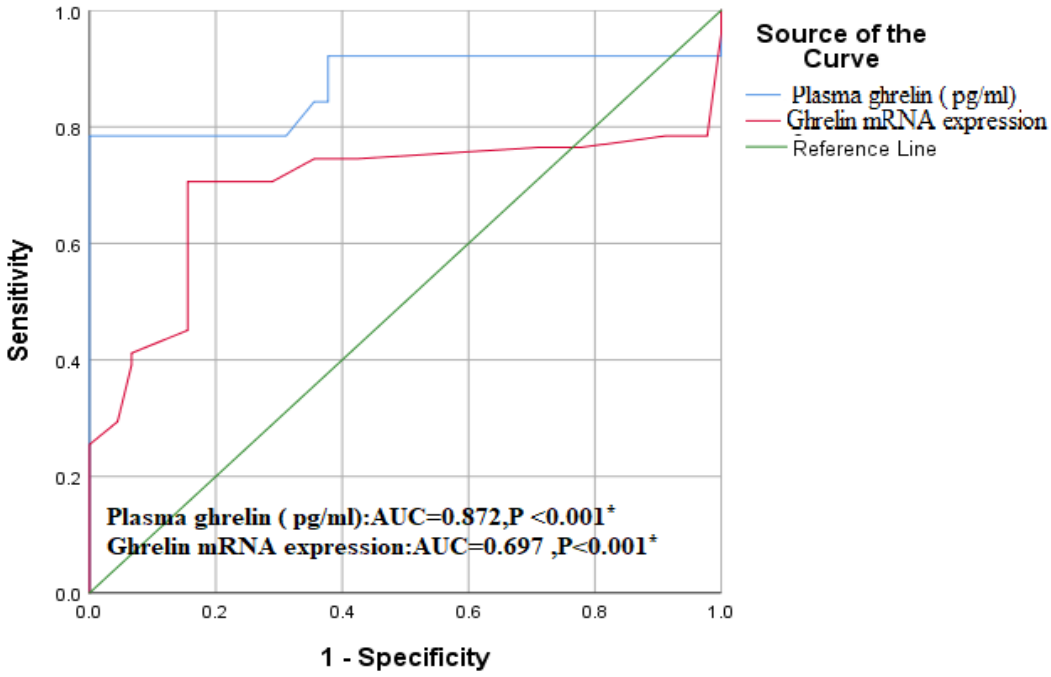

Figure (3): The accuracy of ghrelin plasma and relative expression levels for discriminating ESRD patients from control group by ROC analysis.

DISCUSSION

It has been suggested that malnutrition is the major co-morbid condition of patients on dialysis, thus, assessment of the nutritional status of patients with ESRD should be regularly done as a part of treatment [14]. There is growing evidence that understanding involved mechanisms of pathophysiology in malnutrition development in ESRD is necessary for developing diagnostic, and curative strategies, which can maintain normal nutritional status [15]. Ghrelin is a gastric-derived hormone that acts at the ghrelin receptor to increase appetite and decrease system inflammation, in addition to other regulatory functions [16]. The objective of the current study was to assess the nutritional status of studied subjects with ESRD on regular hemodialysis and to investigate the plasma and mRNA expression levels of ghrelin concerning anthropometric measures, laboratory tests as well as nutritional status.

In the current work, we assessed the nutritional status of Egyptian patients with ESRD by applying subjective global assessment scores (SGA). According to our results patients with ESRD (n=50) stratified to ESRD patients with mild malnutrition (n=10), patients with moderate malnutrition (n=25) and patients with severe malnutrition (n=15).

In the current study, as we assumed, patients with ESRD had significantly higher values of cardiovascular, metabolic, and renal risk factors compared to control group. regarding clinical, anthropometric, and laboratory parameters of nutritional assessment, patients with ESRD had significantly lower values of the triceps skin fold, MAMC, Hb, total protein, albumin, transferrin, and TIBC compared to control groups.

The current explorative study was designed to compare plasma ghrelin levels (pg/ml) in the studied groups. The interesting findings of the current study are that plasma ghrelin levels were significantly higher in ESRD patients with mild and moderate malnutrition compared to the control group. However, the levels of plasma ghrelin were meaningfully lower in ESRD patients with severe malnutrition compared to the control group. **Gupta and his colleagues** [17] detected that levels of digestive ghrelin (DG) increase with declining eGFR. They explained their results by the fact that ghrelin metabolism and secretion are impaired in ESRD. Similar results were observed by **Sharma and his colleagues** [18] where they detected that the levels of acyl ghrelin (AG) were significantly less in the severe category of malnutrition and in the poor appetite group. Similarly, **Khashab *et al.*** [19] detected that adiponectin and ghrelin levels were highest in hemodialysis (HD) patients. Against our results, **Iglesias *et al.*** [20] discovered that patients with ESRD had significantly lower serum ghrelin concentrations than predialysis CKD patients. The discrepancy between the results of the current study and those of previous studies could be a result of differences in sample size and laboratory methods.

The interesting result of our study was that ghrelin mRNA expression levels were significantly higher in ESRD patients with mild and moderate malnutrition compared to the control group. However, the levels of ghrelin mRNA expression were expressively lower in ESRD patients with severe malnutrition compared to the control group. Supporting our results, **Oner-Iyidogan *et al.*** [21] observed that the levels of acyl ghrelin were lowest in ESRD with severe malnutrition and in patients with poor appetite. Similar reports by **Chou *et al.*** [22] identified that low acyl ghrelin is associated with poor nutritional status and loss of appetite.

Ghrelin administered to dialysis patients increased appetite and food intake, induced a sustained positive change in energy balance, and increased food intake in malnourished patients on peritoneal dialysis. Thus, in patients of CKD theoretically low level of AG plays a role in their malnutrition. As a consequence of our studies, we investigated our results by Pearson correlation to assess the correlations between plasma and ghrelin mRNA expression levels as well as clinical, anthropometric, and laboratory parameters of nutritional assessment and we detected significantly positive correlations between plasma and ghrelin mRNA expression levels and serum creatinine and BUN. In addition, there were significant negative correlations between plasma and ghrelin mRNA expression levels with MAMC, Hb, albumin, transferrin, and TIBC.

Even more importantly, we investigated the independent correlations associated with plasma and ghrelin mRNA expression levels in ESRD patients with mild and moderate malnutrition. A linear regression analysis test was done, and our results showed that serum creatinine and transferrin, as well as triceps skin fold, were independently correlated with plasma ghrelin. While, serum creatinine transferrin, triceps skin fold, as well as TIBC, were independently correlated with ghrelin mRNA expression levels.

The interesting finding of the present study was that the diagnostic value of ghrelin plasma and relative expression levels by the ROC test detected that the sensitivities and the specificities of ghrelin plasma and relative expression levels were 92.2% and 63.2% for plasma ghrelin 92.2% and 64.6% for ghrelin relative expression levels respectively.

CONCLUSION

Our results detected that ghrelin plasma and relative expression levels were significantly increased in ESRD patients with mild and moderate malnutrition compared to control subjects. While, ESRD patients with severe malnutrition had lower values of ghrelin plasma and relative expression levels compared to

control subjects. Further future multicenter studies with a bigger sample size are needed to validate our findings.

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Author contribution: Authors contributed equally in the study.

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