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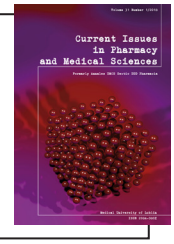

Estimation of Endostatin level in pulmonary arterial hypertension patients and its relation with some parameters

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ABSTRACT

Biomarkers are attractive non-invasive tools for estimating and monitoring pulmonary arterial hypertension (PAH) disease and for predicting survival in patients with PAH; therefore, many studies encouraged the investigation of new biomarkers to facilitate the diagnosis of PAH. Endostatin (ES) is an endogenous inhibitor of angiogenesis. It is produced by proteolytic cleavage of the collagen XVIII that is present in both normal and cancerous tissue. *In vitro* examination shows that ES can manage endothelial cells (EC) physiology in ways that could influence angiogenesis. For example, solvent ES hinders EC movement and prompts improvements of the cytoskeleton that incorporate the loss of Actin stretch strands and central grips. This effect embraces restrictions on the α5β1integrins, Tropomyosin, and putative heparan sulfate proteoglycans. Consequences for the human EC cytoskeleton include Es-induced down-regulation of Mitogen-actuated Protein Kinase (MAPK), Focal Adhesion Kinase (FAK), the Urokinase Plasminogen Activator (uPA) System, and the RhoA GTPase. Human ES has likewise been shown in a few investigations to repress EC multiplication. Moreover, ES-instigated cell cycle capture in the G1 stage is joined by Cyclin D1 down-regulation. Of note, ES blocks the proliferation and organization of endothelial cells into new blood vessels, and in animal studies, ES also inhibits angiogenesis and the growth of both primary tumors and secondary metastasis. ES was initially identified by its capacity to inhibit tumor angiogenesis *in vitro* and also *in vivo*. It can also be found in both healthy and patient' serum, and has been detected in peripheral circulation. ES could be an attractive, non-invasive prognostic marker for some diseases, notably PAH. Therefore, the presented work is aimed at investigating the ES level in blood serum as a biomarker for detection, diagnosis and early treatment of PAH patients. In doing so, the association is ascertained between gender, age, body mass index (BMI), waist circumferences, smoking, types of PAH (primary and secondary) and this potential biomarker is assessed in PAH patients.

ABBREVIATIONS

ANOVA – Analysis of variance; BMI – Body mass index; BMP4 – Bone Morphogenetic protein-4; BMPR2 – Bone Morphogenetic Protein Receptor II; CHD-Congenital Heart Disease; COPD – Chronic obstructive pulmonary disease; CYP1B1 – Cytochrome P450 1B1; DS – Down syndrome; ES – Endostatin; ESR1/Erα – Estrogen Receptor1/ Estrogen Receptor Alpha; HER2 – Human Epidermal growth factor Receptor-2; IPAH – Idiopathic pulmonary arterial hypertension; LHD-Left heart disease; MCTD-Mixed connective tissue disease; MMP – Matrix metalloproteinases; NAPH – Nicotinamide Adenine Dinucleotide Phosphate; PAH – Pulmonary arterial hypertension; PH – Pulmonary hypertension; PPAR-γ – Peroxisome Proliferator-Activated Receptor-gamma; ROS – Reactive oxygen species; SOCE-Stored operated calcium entry; SRC – Scleroderma renal crisis; SSc – Systemic sclerosis; VEGF – Vascular endothelial growth factor family; V6 – Version 6.

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a quiet executioner, which can prompt irreversible changes in pulmonary vascular structure and function, expanding PVR (pulmonary vascular resistance), failure of right ventricular, and demise cases [1]. PAH is known by a resting mean pulmonary artery pressure (PAP) ≥ 25 mmHg, the normal mean PAP at rest is 14± 3 mmHg, and a high limit of normal 20 mmHg [2-4].

The pulmonary hypertension (PH) classification underwent a number of changes prior to the first classification in 1973, which labelled only two categories, primary pulmonary hypertension also known as secondary PH, which depends on the absence or presence of recognizable risk factors or reasons. On PH, a second world symposium was conducted In 1998 in Evian, a city in France, where this cataloguing tried to categorize PH with parallel pathogenesis, therapeutic options and clinical features [5].

The third world symposium that was held in 2003 in Venice did not suggest key changes. Yet, the terms, familial PAH, idiopathic PAH, and associated PAH were presented [6]. In Dana Point in California, the fourth world symposium was held in 2008, an international group of experts consensus revised prior classifications to accurately reproduce published data, and to clarify some fields that were shady. The fifth world symposium was held in 2013 in Nice and presented only slight modifications [6,7].

PAH can come about because of a wide assortment of etiologies, endothelial brokenness prompts an unevenness in the creation of vasoconstrictors versus vasodilators, factors influencing smooth muscle cells, thrombotic arbiters and incendiary cytokines [8].

Therefore, there is movement and expansion of smooth muscle cells into the little precapillary pulmonary arterioles, which regularly do not have a smooth muscle layer, and the irregular nearness of myofibroblasts [9]. This multiplication of smooth muscle cells and myofibroblasts prompts luminal narrowing and a blunted capacity to satisfactorily enlarge, with ensuing increment in the upstream weight. In later stage malady, zones of central and disrupted neovascularization named plexiform sores create [10].

In expansion to medial smooth muscle multiplication, there are self-evident irritations in the extracellular matrix. Tenascin-C, a vast matrix glycoprotein, advances smooth muscle cell multiplication and is exceptionally communicated inside the average layer of renovated vessels in the creature and human investigations [11]. ES is an endogenous angiogenesis inhibitor, which is cut apart from C-terminal of collagen XVIII to become a 20 kDa crunch molecule. O'Reilly and collogues shown ES in 1997 [12].

Moreover, resourceful it can impede endothelial cell proliferation, migration by imminent to α5β1, αvβ3, and αvβ5 integrin receptors [13]. ES contains the broadest anti-angiogenic spectrum reaction though down regulation part of angiogenesis pathway [14].

ES is known to inhibit the endothelial cell proliferation and blockade of VEGF-mediated signaling pathways by interacting with the VEGF receptor of epithelial cells [15-17]. ES structurally has compact globular folding and patches that are surface exposed and arginine-rich [18]. They participate in combining with heparin sulfate, which in turn might be essential for function of endostatin's anti-angiogenic [19,20]. The non-collagenous domain releases ES by the various proteases actions, such as the serine protease elastase, Cathepsin L, and matrix metalloproteinases (MMP) [21]. The released forms could be freely circulating or seen in the vessel wall or in platelets [22].

ES has been fused with HER2 monoclonal antibody, angiostatin, or adversary integrin receptor RGD peptide to restore antitumor efficacy and antiangiogenic movement in multiple cancer types including colon capricorn, ovarian, and pancreatic cancer. [23-27]. Kantola (2016) [15] shows the correlation between ES levels and systemic inflammatory response, but the mechanisms of this linkage remain unknown; ES may be cleaved from non-tumoral tissue when systemic inflammatory response is activated. ES could be released by soluble proteinases such as neutrophil elastase that is known to be increased in the serum of CRC patients [28]. ES levels were elevated in systemic sclerosis (SSc) patients and mixed connective tissue disease (MCTD) are chronic immune mediated disorders becoming complex by vascular organ damage. SSc and MCTD, particularly SSc patients with pulmonary arterial hypertension and scleroderma renal crisis (SRC), and MCTD patients with digital ulcers. Elevated levels of ES have also been related with increased all-cause mortality; ES could show the vascular injury degree in MCTD and SSc patients [29].

Pulmonary fibrosis and pulmonary arterial hypertension (PAH) increase mortality and is mainly driven by them [30]. Microangiopathy seems to be responsible for the lethal organ involvement, such as scleroderma renal crisis (SRC), PAH, gastric antrial vascular actasia and cardiomyopathy, [31] and possibly pulmonary fibrosis [32]. Injury causes hypoxia in healthy tissue vascular inducing proteins in the vascular endothelial growth factor family (VEGF). A variety of cells release VEGF-A such as macrophages, fibroblasts, neutrophils, T cells and endothelial cells, and participates in some neoangiogenesis steps [29].

The strongest inhibitor of VEGF-induced angiogenesis is ES, a peptide that collagen XVIII derives, produced by means of fibroblasts and mainly found in the skin and lungs bottom membranes of the [33]. ES levels increase in SSc and MCTD patients, and related to PAH and SRC in digital ulcers in patients with MCTD patients with SSc [31,32].

High levels of ES are also related to all-cause mortality increment, the dysregulated angiogenesis role in SSc and MCTD and indicate that ES may indicate the vasculopathy degree in such disorders [34]. High levels of ES are also related to all-cause mortality increment, the dysregulated angiogenesis role in SSc and MCTD and indicate that ES may indicate the vasculopathy degree in such disorders [34]. The relationship of a potent angiostatic factor, ES, with disease severity and mortality in PAH, serum ES correlated with poor functional status, decreased exercise tolerance, and invasive hemodynamic variables. Furthermore, serum ES was a strong predictor of mortality [35,36].

Tumor angiogenesis is inhibited by ES by both upregulating factors of anti-angiogenic, such as thrombospondin

and downregulating the factors of pro-angiogenic, such as (VEGF) factor of vascular endothelial growth and VEGFR-2 [14]. ES also induce endothelial cell apoptosis by down regulating the anti-apoptotic B-cell lymphoma-2 (Bcl-2) [37].

MATERIALS AND METHODS

Patients & healthy group

The study was applied on 88 people aged 30-69 years; 67 PAH patients group and 21 healthy group. The samples were collected from echocardiography unit in Cardiac Centre of AL-Sader Teaching Hospital in AL-Najaf AL-Ashraf province /Iraq, during the period from December 2016 to May 2017. The PAH patients group are divided into subgroups according to gender (male and female), age (30-39y, 40-49y, 50-59y and 60-69y), body mass index (BMI) types (normal weight ,over weight and obese weight), waist circumferences (WC) types (70-80 cm , 81-90 cm, 91-100 cm, 101-110 cm and 111-120 cm), smoking (nonsmokers, smokers), primary and secondary of PAH and secondary types of PAH diseases (valvular disease, chronic obstructive pulmonary disease (COPD), congenital heart disease (CHD), Left Heart Disease systolic dysfunction or diastolic dysfunction (LHD sys or dia) and pulmonary embolism) and grade (mild, moderate and severe).

The healthy group are composed of 21 persons appear healthy; they are divided into subgroups according to gender, age, body mass index types, waist circumferences types and healthy nonsmoking, a full history of each subjects was recorded.

Exclusion criteria. The healthy group should have no history of heart disease, PAH, thyroid disorders, chronic liver disease, diabetes mellitus, cancer, renal disorders, anemia, myocardial infarction (MI) and acute infections. The healthy group also entered to echocardiography unit to evaluate the presence of PAH or any disease related with PAH.

Ethical statement. The ethics committee for human of AL-Sader Teaching Hospital in AL-Najaf AL-Ashraf province /Iraq approved protocol.

Collection of Blood samples

In the study, 5 ml of venous blood are drawn from the PAH patients and healthy group between 9-11 a.m. from ante cubital venipuncture using a disposable needle and plastic syringes. The blood was left at room temperature for 10 min to clot in the gel tube. The serum was then isolated after centrifugation at 3000 rpm for 15 min. and subsequently separated and transposed into new disposable tubes Eppendorf tubes by micropipette and stored at -20°C.

Body mass index (BMI)

BMI is calculated by a person's weight (in kg) being divided by the square of his/her height.

$$BMI = Weight\ (kg)/(Height\ m)^2$$

BMI that ranges from 18.5-24.9 kg/m² is normal, while 25-29.9 kg/m² is over weight. Any figure larger than (30 kg/m²) is obese [38].

Waist circumferences (WC)

The measuring of (WC) must be from the top of the iliac crest and the lower margin of the least palpable rib at the midpoint and done by tape (stretch-resistant). The normal measurement for men is 102 cm (40 in), while that for women is 88 cm (35 in) [39].

Primary and Secondary types

Primary type is a disease with no underlying cause. It comes in two forms; one is called 'familial' – a disease that runs in families, while the second form is called 'idiopathic'. The secondary type is one with high pressure in the pulmonary vessels due to some other underlying disease, the most common being valvular heart disease, chronic obstructive pulmonary disease (COPD), congenital heart disease (CHD), Left Heart Disease (LHD) systolic dysfunction or diastolic dysfunction (LHD sys or dia) and pulmonary embolism [40].

Grades of PAH

The patients were divided into grades of disease (Mild, moderate and severe types) depending on echocardiographic results. Herein, assessment of mean PAH is classified into mild at 25-35 mmHg, moderate at 35-45 mmHg and severe at more than 45 mmHg. It is also graded through measuring the pressure gradient via tricuspid valve regurgitation and via pulmonary valve regurgitation [41,42].

Biomarker measurement

The specific kit for measuring human Endostatin (ES) level in serum was supplied by (Elabscience Catalog No: E-EL-H0063/96T). The sensitivity range of this kit is between 0-10 ng/ml, according to user manual.

Determination of serum Endostatin level

Test principle

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been precoated with an antibody specific to Human ES. Standards or samples are added to appropriate micro ELISA plate wells and combined with the specific antibody. Then, biotinylated detection antibodies specific for Human ES and Avidin-Horseradish Peroxidase (HRP) conjugate are added to each micro plate well successively and incubated. After incubation, free components are washed away. Subsequently, Substrate Reagent is added to each well. Here, only those wells that contain Human ES, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme substrate reaction is terminated by adding Stop Solution and appears yellow in color. The optical density (OD) can be measured with spectrophotometry at a wavelength of 450 nm ±2 nm. The OD value is proportional to the concentration of Human ES. The concentration of Human ES in samples can be calculated by comparing the OD of the samples with the standard curve.

Components

Item	Specifications	Storage
Micro ELISA Plate	8 wells × 12 strips	4°/-20°
Reference Standard	2 vials	4°/-20°
Reference Standard & Sample Diluent	1vial 20mL	4°
Concentrated Biotinylated Detection Ab	1vial 120µL	4°/-20°
Biotinylated Detection Ab Diluent	1vial 10mL	4°
Concentrated HRP Conjugate	1vial 120µL	4°(shading light)
HRP Conjugate Diluent	1vial 10mL	4°
Concentrated Wash Buffer (25×)	1vial 30mL	4°
Substrate Reagent	1vial 10mL	4°(shading light)
Stop Solution	1vial 10mL	4°
Plate Sealer	5pieces	
Manual	1 copy	
Certificate of Analysis	1 copy	

Assay procedure

1. Add 100 µL standard or sample to each well. Incubate for 90 min at 37°C.
2. Remove the liquid. Add 100 µL Biotinylated Detection Ab. Incubate for 1 hour at 37°C.
3. Aspirate and wash 3 times.
4. Add 100 µL HRP Conjugate. Incubate for 30 min at 37°C.
5. Aspirate and wash 5 times.
6. Add 90 µL Substrate Reagent. Incubate for 15 min at 37°C.
7. Add 50 µL Stop Solution. Read at 450 nm immediately.
8. Calculation of results.

Statistical analysis

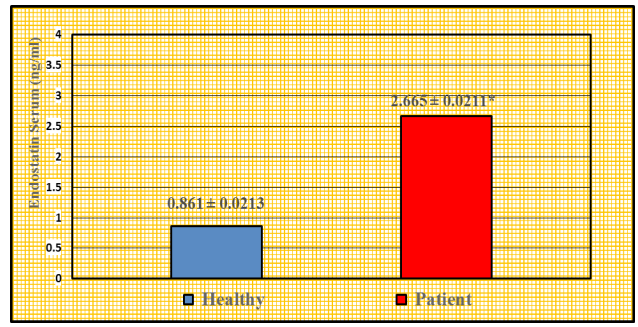
Graphpad Prism v6 windows software packages are used to analyze the data of the present study (Version 6.01, 2012 for Windows 2010), data were ordered as Mean±Standard deviation (SD).

Unpaired sample t-test was used for the comparison between two groups and one-way ANOVA test was used for the comparison among subdivided groups in the measured parameters, p value < 0.05 was used as a level of statistically significance. All the figures of this study were constructed by using the EXCEL program of Microsoft Office 2010.

RESULTS

Comparison of ES serum level between the PAH patients group and the healthy group

The results exhibit a significant increase (p > 0.05) in the ES serum level of PAH patients (2.665±0.0211 ng/ml) compared with that of the healthy group (0.861±0.0213 ng/ml), as shown in Figure 1.

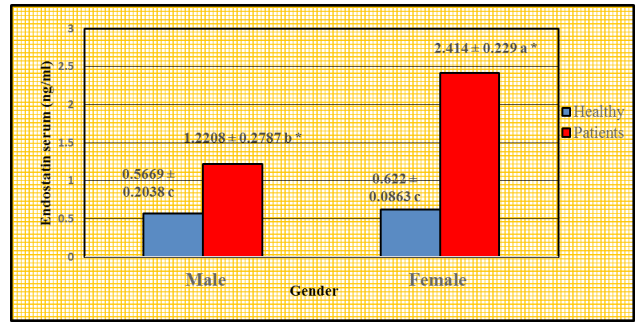


* – Represents significant differences at (p>0.05) between means

Figure 1. Comparison of ES serum level between PAH patients group and healthy group

Comparison of ES serum level between males and females of the PAH patients group and healthy group

The result (Fig. 2) show a significant increase (p>0.05) in the ES serum level of the female PAH patients group (2.414±0.229 ng/ml), as compared with the male PAH patients group (1.2208±0.2787 ng/ml) and also as compared with males and females of the healthy group (0.5669±0.2038 ng/ml, 0.622±0.0863 ng/ml, respectively).

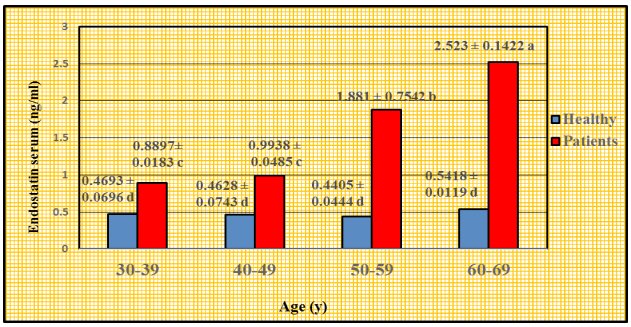


The dissimilar letters represent significant differences (p>0.05) between different groups. The similar letters represent non-significant difference
* – Represents significant differences at (p>0.05) between means

Figure 2. Comparison of ES serum level between males and females of the PAH patients' group and the healthy group (according to gender)

Comparison of ES serum level among different age subgroups of the PAH patients group and the healthy group

The results shown in Figure (3) indicate that there are significant increases (p>0.05) in ES serum levels within the different age subgroups of the PAH patients group (2.523±0.1422 ng/ml, 1.881±0.7542 ng/ml, 0.9938±0.0485 ng/ml and 0.8897±0.0183 ng/ml, respectively), when compared with healthy group counterparts (0.5418±0.0119 ng/ml, 0.4405±0.0444 ng/ml 0.4628±0.0743 ng/ml and 0.4693±0.0696 ng/ml, respectively) for the ages (60-69y), (50-59y), (40-49y) and (30-39y), respectively. The age subgroup (60-69y) shows the highest significant increase (p>0.05) in ES serum level (2.523±0.1422 ng/ml) when compared with the other age subgroups, while the results show non-significant differences between the PAH patients subgroups of ages (30-39y) and (40-49y), respectively (Fig. 3).

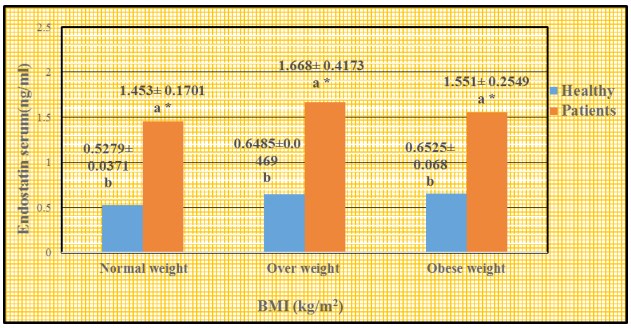


The dissimilar letters represent significant differences (p>0.05) among different ages. The similar letters represent non-significant differences

Figure 3. Comparison of ES serum level among different age subgroups of the PAH patients group and the healthy group

Comparison of ES serum level among the PAH patients group and the healthy group according to BMI (normal weight, over weight, obese weight)

The results indicate there are non-significant differences in ES serum level within the PAH patients group according to BMI (normal weight, over weight, obese weight), but there are significant increases (p>0.05) in ES serum level in the PAH patients group (1.453±0.1701 ng/ml, 1.668±0.4173 ng/ml and 1.551±0.2549 ng/ml, respectively), when compared to their healthy group counterparts (0.5279±0.0371 ng/ml, 0.6485±0.0469 ng/ml and 0.6525±0.068 ng/ml, respectively) (Fig. 4).

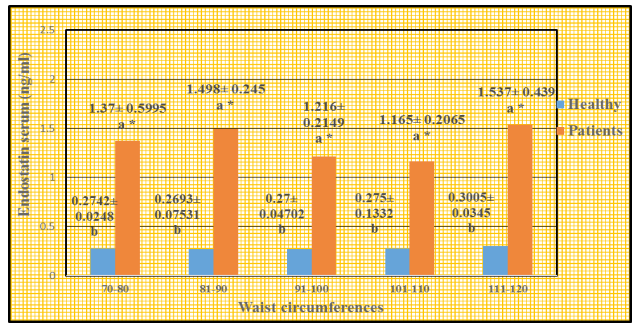


The dissimilar letters represent significant differences (p>0.05) between different groups. The similar letters represent non-significant difference
* – Represents significant differences at (p>0.05) between means

Figure 4. Comparison of ES serum level among the PAH patients group and the healthy group according to BMI (normal weight, over weight, obese weight)

Comparison of ES serum level among the PAH patients group and the healthy group according to waist circumferences.

The results in Figure 5 reveal non-significant differences between the whole of the PAH patients group according to waist circumferences. However, the results show significant increases (p>0.05) in ES serum level in the patients group (1.37±0.5995 ng/ml, 1.498±0.245 ng/ml, 1.216±0.2149 ng/ml, 1.165±0.2065 ng/ml and 1.537±0.439 ng/ml, respectively) compared with their healthy group counter-parts (0.2742±0.0248 ng/ml, 0.2693±0.07531 ng/ml, 0.27±0.04702 ng/ml, 0.275±0.1332 ng/ml and 0.3005±0.0345 ng/ml, respectively) according to waist circumferences (70-80, 81-90, 91-100, 101-110 and 111-120 cm).

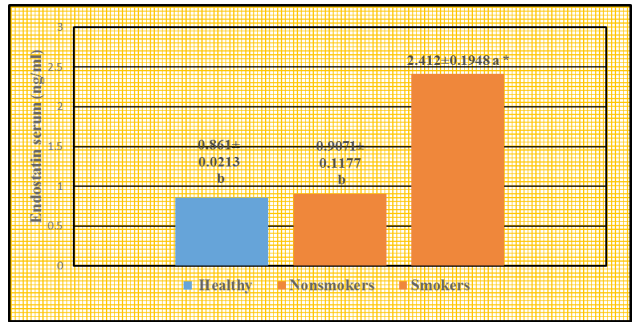


The dissimilar letters represent significant differences (p>0.05) between different groups. The similar letters represent non-significant difference
* – Represents significant differences at (p>0.05) between means

Figure 5. Comparison of ES serum level among the PAH patients group and the healthy group according to waist circumferences

Comparison of ES serum level between the healthy group (nonsmokers) and the PAH patients' group (nonsmokers and smokers).

The results shows significant increase (p>0.05) in ES serum level in the PAH patients smokers subgroup (2.412±0.1948 ng/ml) as compared to the PAH patients nonsmokers subgroup and between it and the healthy group nonsmokers subgroup (0.9071±0.1177 ng/ml and 0.861±0.0213 ng/ml respectively), but there is a non-significant difference between the healthy group and the nonsmokers PAH patients group (Fig. 6).

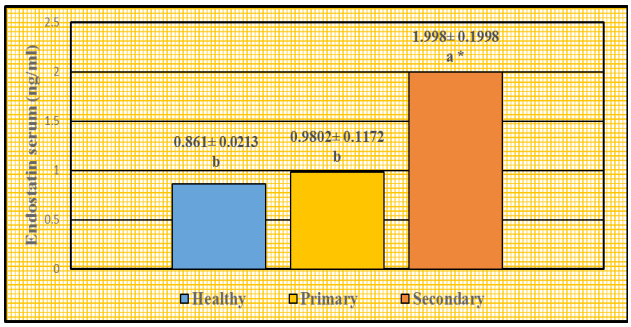


The dissimilar letters represent significant differences (p>0.05) between different groups. The similar letters represent non-significant difference
* – Represents a significant difference at (p>0.05) between means

Figure 6. Comparison of ES serum level between the healthy group nonsmokers subgroup and the PAH patients subgroups of nonsmokers and smokers

Comparison of ES serum level among the healthy group and the primary and secondary PAH patient subgroups

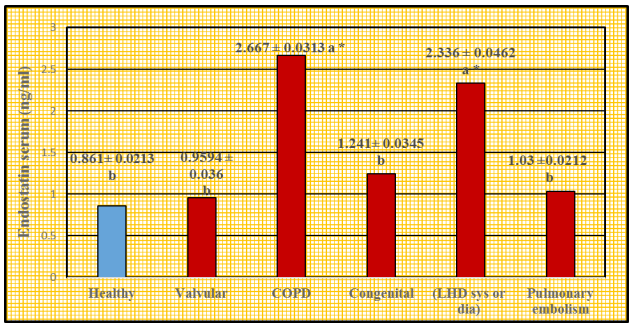
The results revealed in Figure 7 indicate that there are significant increases (p>0.05) in ES serum level within the secondary PAH patient subgroup (1.998±0.1998 ng/ml) when compared with the primary PAH patient subgroup (0.9802±0.1172 ng/ml) and with the healthy group (0.861±0.0213 ng/ml), but there is non-significant differences between the healthy group and the primary PAH patients group**.**



The dissimilar letters represent significant differences (p>0.05) between different groups. The similar letters represent non-significant difference
* – Represents a significant difference at (p>0.05) between means

Figure 7. Comparison of ES serum level among the healthy group, the primary and secondary PAH patient subgroups

Comparison of ES serum level among the PAH patients groups of diverse secondary diseases and the healthy group.



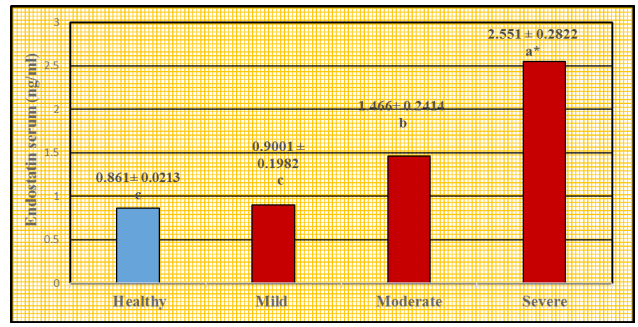
The dissimilar letters represent significant differences (p>0.05) between different groups. The similar letters represent non-significant difference
* – Represents significant differences at (p>0.05) between means

Figure 8. Comparison of ES serum level among PAH patient subgroups of diverse secondary diseases and the healthy group

The results in Figure 8 indicate a significant increase (p>0.05) between the PAH patients subgroups of COPD and LHD sys or dia (2.667±0.0313 ng/ml and 2.336±0.0462 ng/ml, respectively) when compared with the healthy subgroups of valvular, congenital and pulmonary embolism (0.861±0.0213 ng/ml, 0.9594±0.036 ng/ml, 1.241±0.0345 ng/ml and 1.03±0.0212 ng/ml respectively). The results show non-significant differences between COPD and LHD sys or dia PAH patient subgroups, and demonstrate non-significant differences among healthy subgroups of valvular, congenital and pulmonary embolism.

Comparison of ES serum level between different PAH patient grade subgroups and the healthy group

The results in Figure 9 shows a significant increase (p>0.05) among the PAH patient grade subgroups of severe (2.551±0.2822 ng/ml), moderate (1.466±0.2414 ng/ml) and mild (0.9001±0.1982 ng/ml, respectively. The results also demonstrate a significant increase (p>0.05) in severe and moderate (2.551±0.2822 ng/ml and 1.466±0.2414 ng/ml) grade subgroups when compared to the healthy group (0.861±0.0213 ng/ml), but there is a non-significant difference between the healthy group and the mild PAH patients group.



The dissimilar letters represent significant differences (p>0.05) between different groups. The similar letters represent non-significant difference
* – Represents a significant difference at (p>0.05) between means

Figure 9. Comparison of ES serum level between different PAH patient grade sub groups and the healthy group

DISCUSSION

The results exhibit significant increases (p>0.05) in the ES serum level of PAH patients when compared with healthy counterparts as showed in Figure 1. The present study is in agreement with a study of PAH disease that reported that ES serum level increased in PAH patients. ES is a potent inhibitor of angiogenesis with the capacity to induce endothelial cell apoptosis and inhibit endothelial cell proliferation and migration. Here, a loss of function variant in the gene encoding ES was linked to altered ES serum levels in PAH. What is more, the ES genotype is independently associated with mortality in PAH [36].

Another study confirmed the increased ES serum level in arterial hypertension patients. This comes about because of the imbalance between proangiogenic to antiangiogenic factors in addition to increases in the portent antiangiogenic factors ES and decreases in the level of proangiogenic factors. These are the main characteristics of PAH disease wherein, the increase peripheral resistance in PAH is brought about by a decrease in the synthesis of proangiogenic mediator and an increase in antiangiogenic factor production which in turn leads to microvascular rarefaction [43].

A study of Reiseter *et al*. (2015)[29] demonstrated that ES levels are elevated in (SSc) and (MCTD) patients and are associated with PAH and scleroderma renal crisis (SRC). The role of dysregulated angiogenesis in SSc and MCTD suggests that ES could reflect the degree of vasculopathy in these disorders [29]. What is more, myocardium synthesized ES is an antiangiogenic peptide that is highly detectable in the serum of patients with heart failure [44].

Another study has documented elevated ES levels in the blood serum of patients infected with PAH which is associated with the severity of the disease. Here, less oxygen delivery is felt by the myocardial that participates in the transition from right side heart adaptation to failure [36].

The study of Galambos *et al*. (2016)[45] concluded that lung anti-angiogenic factors, including increased ES expression and decreased growth vessel of fetal lung of those with Down syndrome (DS) or trisomy 21, lead to impaired lung vascular growth and signal alveolarization, which is a high risk factor for PAH [45].

The results shown in Figure 2 illustrate a significant increase (p>0.05) in the ES serum level of female PAH

patients group as compared with the male PAH patients group and also compared with males and females of the healthy group. The current study is consistent with a study of Badesch *et al*. (2010) [46] who suggest that familial and idiopathic forms of PAH are likely to present in females up to four times as often than in males [46]. The sex hormones (particularly both the endogenous and exogenous estrogens) are high risk factors and are implicated in PAH. Indeed, an experimental study on female mice postulated that elevations of estrogen in the lung of female patients are association with aromatase, ESR1/Erα and ES levels and should be considered as generating a predisposition to Porto pulmonary hypertension [47,48].

Another explanation for the elevation of estrogen and ES is that this is due to alterations in the CYP1B1 gene to which female heritable PAH patients are very susceptible [49]. Estrogens have a protective effect on females in improving right ventricular contractility [50]. High levels of estrogen are associated with elevations in ES level because the estrogen and ES engender vasoconstriction and antiangiogenic induction of endothelin-1 – which is considered as being a potent mitogen and vasoconstrictor in pulmonary arteries – especially after menopause.

The results displayed in Figure 3 indicate there are significant increases ($p>0.05$) in ES serum levels within the different ages subgroups of the PAH patients group, when compared with the healthy group for the ages (60-69y), (50-59y), (40-49y) and (30-39y), respectively. Here, the age subgroup of (60-69y) shows the highest significant increase ($p>0.05$) in ES serum level as compared with the other ages, while the results show non-significant differences between the PAH patient subgroups of ages (30-39y) and (40-49y), respectively (Figure 3). The present study in agreement with the study of Asai *et al*. (2002) [50] that demonstrated an existing imbalance between VEGF/ES in sputum and a higher positive correlation between VEGF/ES in elderly asthmatic patients. Here the changes in these ratios mirror changes in the vascularity of airway mucosa which leads to PAH. Furthermore, the changes in the homeostatic regulation between angiogenic and antiangiogenic in the higher age subgroups may be related to the increase inflammation and vascular growth proliferation of new blood vessels that is correlated with morphogenic changes in the mucosal PAH airway [51]. In addition, Ling *et al*. (2012) [52] reported that younger patients aged > 50 years have better survival rates that older patients. The high mortality rate and incidence of PAH elder patients are due to increase in right ventricular heamodynamic function, exercise limitation and endothelial dysfunction due to increase ES and endothelin-1 levels that stimulate vasoconstriction and proliferation of smooth muscle cells [52].

The increase production of reactive oxygen species (ROS) during hypoxia may also be related to enhanced ES levels in the elderly. Some studies suggest that the level of NAPH-oxidase is activated and that the generation of hydrogen peroxide (H_2O_2) was mediated in the lung and leads to pulmonary vascular contraction [53,54].

The results also show a significant increase ($p>0.05$) in ES serum level between the PAH patient subgroups of smoker and nonsmoker and the healthy group (Fig. 6). According to researchers, smoking exposure induces pulmonary vascular remodeling that leads to increased stored operated calcium entry (SOCE) and, it turn to higher expression of BMP4, PPAR-γ and ES level [55]. It is suggested that cigarette-smoke induces PAH by up-regulation of BMP4 and BMPR2. Moreover, ES level and high expression are shown not only in the blood stream, but also in the whole lung [56].

Figure 7 reveals there are significant increases ($p>0.05$) in ES serum level within secondary PAH patients groups as compared with the primary PAH patient subgroups and the healthy group. The study of Damico *et al*. (2015)[36] is in agreement with the present study, which confirms that serum ES level is a predictor of survival in PAH, and a strong predictor of mortality [36], therefore, its level increases in the development of secondary PAH. ES is a potent angiostatic peptide and may be elevated in secondary PAH due to increased proliferation and the angiogenesis process.

The results displayed in Figure 8 indicate a significant increase ($p>0.05$) between the PAH patient subgroups of chronic (COPD) and left heart disease (LHD sys or dia) compared with the healthy subgroups of valvular, congenital and pulmonary embolism. The results give non-significant differences within both patient groups with regard to COPD and LHD (sys or dia) alone and non-significant differences among healthy subgroups of valvular, congenital and pulmonary embolism. Some studies have been shown ES levels to be elevated in LHD (sys or dia) that is associated with PAH in circulation, pericardial space and myocardial tissue that may be correlated with diminished collateral circulation within the heart [57,58].

The serum ES source is in the myocardium, therefore, ES may be detected in situations of heart disease and damage in the myocardium tissue. Both COPD and hypoxia have been associated with the major pathogenic mechanism of PAH. Some studies in agreement with this notion have revealed structural abnormalities in the pulmonary arteries in moderate COPD [59,60]. Other studies have demonstrated as antiangiogenic factors, increased bronchial and muscular artery infiltration to CD8+T lymphocytes, as well as elevated ES [61]. Herein, the changes in the ratio of VEGF/ES may play an important role in the pathogenesis of intimal cell proliferation in the pulmonary arteries of patients with COPD, Furthermore, ES may alter the vascular remolding in the human lung, and the inhibition of VEGF under hypoxia conditions can lead to pulmonary vascular lesions similar to PAH.

The results shown in Figure 9 demonstrate a significant increase ($p>0.05$) among the different grade subgroups (severe, moderate, mild) of the secondary and primary PAH group, and the healthy group. The present study is in accordance with Damico *et al*. (2015) [36], who demonstrated that serum ES level is correlated with disease severity and can be used to predict the outcome of patients with PAH. Additional observations provide evidence that the gene encoding ES (Col18a1- SNP rs1248337) in severe PAH patients may be altered via in-serum ES [62]. What is more, researchers have noted that the disruption of the myocardial- microvascular balance (which has impact on the right ventricle) and decrease RV myocardial perfusion is associated inversely by elevation of ES and RV systolic function [63].

The results also indicate there are non-significant differences in ES serum level and the BMI (normal weight, over weight and obese weight) and WC of the PAH patients group (Figs. 4 and 5, respectively). A study of Ashwell and Gibson (2017) [64] in cardiometabolic risk factors in adults is in agreement with the present study. Hence, we recommend that the WC and BMI be adjusted to show that having a high WC even in the healthy range of BMI is not necessary an emergency [65,66]. BMI and WC of PAH patients vary due to heredity, physiological metabolism factors, type of food intake, type of physical activities performed by the individual on a daily basis and the period of the disease. Therefore, we find that PAH patients who are either obese weight, over-weight or of normal weight and of diverse waist circumferences (large or small). In addition, there is no significant relation between ES level and the BMI, WC criteria.

Such non-significant differences in ES relations with BMI and WC have also been seen by Taichman and Mandel, (2007) [67] and Lau *et al*. (2017) [68] who demonstrated that in the absence of relation between obesity and BMI, obesity is not to be considered a risk factor for PAH. Burger *et al*. (2011) [69] have also shown no significant differences in BMI between PAH patients. Here, the obese percentage was ≥ 30%, normal weight was > 20.8%, and underweight was <18.5%. However, Taraseviciute and Voelkel (2006) [70] and Leone *et al*. (2009) [71] indicate IPAH with metabolic syndrome co-relates with highly frequency of obesity with lung malfunction, thus they hold that metabolic syndrome is an independent predictor for IPAH. Moreover, Zeng *et al*. (2012) [72] also showed an association between lower BMI as factor for PAH in younger patients and prognosis with IPAH and with mortality.

The current study excluded the factors that affected IPAH, and only PAH patients who were without any related diseases (such as metabolic syndrome, diabetes, thyroid disorder, chronic liver disease and renal disorder) were part of the study population. Therefore, the current results document there is no relationship between BMI and PAH.

CONCLUSION

Endostatin is a potential biomarker for the detection and diagnosis of PAH.

SIGNIFICANCE STATEMENTS

This study is the first clinical study in Iraq.

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