TABLE OF CONTENTS

**Review**
1. Physiological Parameters Affecting the Modulatory Role of Airway Epithelium on Airway Smooth Muscle Responsiveness
   1-8

**Case Report**
2. Renal Salt Wasting Syndrome due to Carboplatin in a Patient with Lung Cancer
   9-12

**Research**
3. Alpha-1 Antitrypsin Gene Polymorphism in the Egyptian Population: Association with Obstructive Lung Diseases
   – Rasha Daabis*, Shaden Muawia, Amal Ahmed, Mohamed El-Shahat, Ahmed Youssef and Tarek Fekry
   13-20

4. Microarray Analysis Identifies Pathways In Progression of Early Stage Lung Adenocarcinoma: The Importance of Focal Adhesion and ECM-Receptor Interactions
   – Susan E Douglas*, Drew C. Bethune* and Zhaolin Xu*
   21-31

5. The Impact of VAP Staff Education on VAP Morbidity and Mortality in Alexandria University
   – Elmenshawy AM, ElbadawyTH, Abu khaber H, Hafez SF, Fayed AM and Ibrahim EH
   32-45
Physiological Parameters Affecting the Modulatory Role of Airway Epithelium on Airway Smooth Muscle Responsiveness

Apostolia Hatziefthimiou*, Molyvdas Paschalis-Adam and Paraskeva Efrosyni

Laboratory of Physiology, Department of Medicine, School of Health Sciences, University of Thessaly, 3 Panepistimiou Str, 41500 BIOSPOLIS Larissa, Greece

*Corresponding author:
Apostolia Hatziefthimiou, MD
Associate Professor
Laboratory of Physiology
Department of Medicine
School of Health Sciences
University of Thessaly
3 Panepistimiou Str 41500 BIOPOLIS Larissa, Greece
Tel. +30 2410 685561
Fax: +30 2410 685555
E-mail: axatzi@med.uth.gr

ABSTRACT
Numerous studies have revealed the significant action of airway epithelium as a non-specific defence mechanism in airways. In addition, epithelial cells release biologically active agents, which modulate airway tone. Importantly, airway epithelium function is influenced by physiological parameters, including the release of endogenous factors, age, gender, load, and bronchi size. The primary goal of this review is to summarize knowledge concerning the effect of the aforementioned parameters on the modulatory role of airway epithelium on airway smooth muscle responsiveness. These effects may be implicated in the pathophysiology of airway diseases like asthma and Chronic Obstructive Pulmonary Disease (COPD).

KEYWORDS: Airways, Epithelium, Nitric oxide.

INTRODUCTION
Respiratory epithelium belongs to the class of ciliated pseudostratified columnar epithelium due to the arrangement of the columnar epithelial cells. Airway epithelium functions as a barrier to potential pathogens and foreign objects and prevents infection by the action of the ciliary escalator. It acts as a non-specific upper airways defence mechanism, that entraps particles and other inhaled material in mucus, and transports them away from the lungs. Efficient mucociliary transport is the result of the co-ordination of three airway epithelial functions, i.e. mucus secretion, ciliary beat and ion and fluid transport. Another important function of airway epithelium is its ability to produce endogenous biologically active substances like Nitric Oxide (NO), prostanoids, and endothelin. It is also worth mentioning that the function of airway epithelium is influenced by physiological parameters, like age, gender, load, and bronchi size.

Airway Smooth Muscles (ASM), whether contracted or relaxed, affect airway diameter and thus air flow to alveoli where gas exchange occurs. Excessive responsiveness of ASM to contractile agents is often characteristic of chronic respiratory diseases, with asthma being a typical example. This over-responsiveness results in airway obstruction and decline of airway flow. Remarkably, a common finding in asthma is epithelium damage, inflammation and in some cases airway remodelling. Moreover, epidemiological data suggest that the incidence of asthma becomes higher in females than males with the onset of puberty, and that this tendency prevails throughout the reproductive years.

These observations triggered research interest toward the modulatory role of airway epithelium on ASM, in connection with the action of NO, prostanoid, cholinergic agents, mediators of inflammation and growth factors, but also in connection with airway size, animal age or gender and the initial load applied on airway smooth muscle.

In the following paragraphs, we discuss the impact of the above factors on the modulatory role of airway epithelium on ASM, as well as the possible implications on the pathophy-
Pulmonary Research and Respiratory Medicine

-siology of airway diseases like asthma and Chronic Obstructive Pulmonary Disease (COPD) (Table 1).

<table>
<thead>
<tr>
<th>Endogenous factors</th>
<th>Effect on epithelium</th>
<th>Possible implication in airway diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>Promotes the survival of epithelial cells(^{11})</td>
<td>Respiratory system is considered an alternative route for insulin administration for the treatment of type 1 diabetes mellitus(^{12})</td>
</tr>
<tr>
<td></td>
<td>Participates in epithelium restoration integrity after its damage(^{12})</td>
<td>Low incidence of asthma in patients with diabetes mellitus(^{12})</td>
</tr>
<tr>
<td></td>
<td>Stimulates NO release from epithelial cells(^{13})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Relaxes precontracted airways(^{13})</td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>Causes: H(_2)O(_2) production from epithelial cells(^{16}), mucus release and swelling of mucosa(^{16}) and NO release from epithelial cells(^{13,17,18})</td>
<td>Possible contribution to the increased airways responsiveness observed in asthma due to epithelium damage and inflammation</td>
</tr>
<tr>
<td></td>
<td>Relaxes precontracted airways(^{18})</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Stimulates NO release from epithelial cells(^{20,68})</td>
<td>Possible contribution to the increased airways responsiveness observed in asthma due to epithelium damage and the increased acetylcholine release(^{19})</td>
</tr>
<tr>
<td>Age</td>
<td>Affects the capacity of airway epithelium to produce NO(^{20,60})</td>
<td>Epithelium-derived NO has an important role in the regulation of airway tone in early newborn life(^{20,71})</td>
</tr>
<tr>
<td>Gender</td>
<td>In rabbit trachea testosterone relaxes precontracted ASM in an epithelium-dependent way(^{11})</td>
<td>Gender differences in the incidence of asthma</td>
</tr>
<tr>
<td></td>
<td>Testosterone serum levels are depressed in patients with respiratory failure,(^{76}) cystic fibrosis,(^{77}) hypoxic pulmonary fibrosis(^{78}) and COPD(^{19})</td>
<td></td>
</tr>
<tr>
<td>Airway size</td>
<td>Variations in the distribution of acetylcholinesterase(^{28,59}), (^{59}) Depends on animal species. E.g. in canine airways, epithelium mainly modulate the responsiveness of the large airways while sheep airways, epithelium integrity affects mainly the responsiveness of small airways(^{69})</td>
<td>Regional differences in ASM responsiveness to contractile agents</td>
</tr>
<tr>
<td></td>
<td>Non-homogeneous distribution of bronchoconstriction observed in COPD and asthma</td>
<td></td>
</tr>
<tr>
<td>Load</td>
<td>Epithelium responds to stretch by modulating epithelial NO synthase activity, NO production and ASM responsiveness to acetylcholine at increased load(^{20,62})</td>
<td>Loss of the protective effect of deep inspiration in asthma(^{50,42}) and patients with COPD(^{13})</td>
</tr>
</tbody>
</table>

**Table 1:** The effects of different physiological parameters on the modulatory role of epithelium on Airway Smooth Muscle (ASM) and the possible implication of these effects in airway diseases.

**ENDOGENOUS FACTORS: INSULIN, HISTAMINE, ACETYLCHOLINE**

Endogenous factors like insulin, histamine or acetylcholine cause airway contraction. On the other hand, they act on epithelial cells and cause the release of biologically active mediators, mainly NO, that relax ASM and as a result limit excessive airway contraction.

Insulin is the major modulator of blood glucose levels exhibiting also growth factor activity on many cell types including ASM cells. Namely, it promotes ASM cell proliferation, via activation of the Phosphatidylinositol 3-kinase (PI3K) pathway,\(^{6}\) switches the ASM phenotype to contractile,\(^{23,24}\) induces the release of contractile prostanooids from sources other than epithelium\(^{20}\) and increases the responsiveness of ASM to contractile agents.\(^{7}\) Moreover, available data suggest that in airways, insulin promotes the survival of epithelial cells\(^{11}\) and participates in epithelium restoration integrity\(^{12}\) after its damage. Additionally, insulin may stimulate NO release from epithelial cells and thus cause relaxation of precontracted airways.\(^{13}\)

Histamine, a classical inflammatory agent, induces airway contraction.\(^{15}\) Histamine further affects airway epithelium as it promotes H\(_2\)O\(_2\) production from epithelial cells of bronchi,\(^{15}\) mucus release and swelling of mucosa.\(^{36}\) In fact, the effect of histamine on ASM contraction is mediated by the release of biologically active molecules like NO from epithelial cells and thus, depends on epithelium integrity.\(^{14,17,18}\) Studies on vessels demonstrate that histamine stimulates endothelial cell H\(_1\) receptors\(^{19}\) by increasing epithelial NO synthase phosphorylation and activity.\(^{20}\)

Acetylcholine, the neurotransmitter released from postganglionic parasympathetic vagus nerves, induces airways contraction via Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) entry from extracellular space.\(^{21,22}\) Additionally, acetylcholine is also released from epithelial cells\(^{23}\) and can promote the chemoattract of monocytes and neutrophils\(^{24,25}\) via, at least in part, the release of interleukin-8.\(^{26}\) ASM express mainly M\(_{2}\) and M\(_{3}\) muscarinic receptors.\(^{27}\) Acetylcholine, as well as other muscarinic agonists, may induce proliferation of ASM cells that
depends on the activity of the MAPK and PI3K pathways, either alone, or in combination with growth factors. In vitro studies demonstrate that the airway epithelium stimulates the breakdown of acetylcholine. In addition, acetylcholine seems to stimulate NO release from the epithelium and its mechanical removal increases airway responsiveness to acetylcholine.

AGE

Animal studies suggest that increased age decreases airway responsiveness to contractile agents, and increases their capability to relax. These age-dependent alterations in airway responsiveness are attributed to changes in airway architecture and organization, to the maturation of the non-adrenergic non-cholinergic system and to increased activity of acetylcholinesterase.

Evidence suggests that age may affect the release of biologically active factors, mainly NO, from the airway epithelium. The three NO synthase isoforms are expressed in airways, but their levels remain unchanged during life. However, the capacity of airway epithelium to produce NO seems to increase with age. Namely, in rabbits (Figure 1, our unpublished results) and pigs, age affects the acetylcholine-induced NO production. Specifically, contractility studies were performed on tracheal strips obtained from young (four weeks old) or adult (eight weeks old) rabbits in the presence of 10^-9 to 10^-3 M of acetylcholine. In adult rabbits epithelium damage increases acetylcholine-induced contractions. On the contrary, these experiments revealed that in young rabbits the mechanical removal of epithelium (Figure 1A, our unpublished results) as well as the treatment of preparations with the inhibitor of NO synthase NG-nitro-L-arginine methyl ester (L-NAME), the precursor of NO formation L-arginine or the inhibitor of cyclooxygenase indomethacin had no effect on ACh-induced contractions (Figure 1B, our unpublished results).

Histological studies reveal that the expression of M3 receptors, which are involved in acetylcholine mediated NO release from the epithelium, increase with age.

GENDER

Epidemiological data indicate a role of sex hormones in the etiology of some chronic airway diseases, in particular, asthma. Gender differences in the incidence of asthma are attributed mainly to differences in the immune response, as testosterone is considered to be immunosuppressant while female sex steroids proinflammatory. Moreover, studies on blood vessels have provided evidence that testosterone may exert direct effects on smooth muscle.

Immunohistochemistry studies show that ASM obtained from male rabbits express classical androgen and estrogens receptors. Similarly, immunofluorescence experiments performed in our laboratory revealed that rabbit ASM cells express Androgen Receptors (ARs). In the majority of cells ARs are cytoplasmic. However, in a few ASM cells ARs are also present in the cell nucleus (Figure 2, our unpublished results).

During embryonic life, sex hormones contribute to growth and maturation of the respiratory system. Androgens seem to delay the maturation of embryonic lungs and might be involved in the pathogenesis of the Acute Respiratory Distress Syndrome (ARDS) that has increased incidence in male newborn. In vitro studies revealed that both androgens and estrogens may affect, via classical receptors, the proliferation of ASM. Also, sex hormones modulate directly the responsiveness of airway smooth muscle to contractile agents via a non-genomic pathway. Namely, in rabbit trachea 17β-estradiol relaxes precontracted airways in an epithelium independent way. On the other hand, testosterone may increase vagal activity and thus contract rab-
load

During the tidal action of breathing load fluctuations are imposed continuously on ASM that undergo shortening and lengthening. Stress and strain can both be the mechanical signals involved in mechanosensitive modulation of ASM activation depending on the applied contractile stimuli. The mechanisms involved comprise ASM stiffness and extensibility, alterations in intracellular Ca²⁺ concentration and regulation of molecules involved in contractile protein activation. Despite the above involved mechanisms, the epithelium may also have a modulatory role in the mechanosensitive modulation of ASM responsiveness. To be precise, studies suggest that airway epithelium modulates ASM responsiveness to acetylcholine at increased load. This effect is mediated at least in part via NO release from epithelial cells.

The involved pathway comprises the calcium dependent activation of epithelial NO synthase. As epithelium responds to stretch by modulating epithelial NO synthase activity, and thus NO production with a consequent reduction of airway responsiveness, this protective mechanism could be impaired in epithelial damage seen in airways diseases in particular asthma.

CONCLUSION

In conclusion, airway epithelium has a significant modulatory role in ASM responsiveness to contractile agents. This role depends on age, gender, bronchi size and load. Also, endogenous released substances like hormones (insulin, sex hormones), inflammatory factors (histamine) and neurotransmitters (acetylcholine) act on airway epithelial cells, induce NO release and limit airway contraction. These factors may contribute to the pathophysiology of some airway diseases like asthma and Chronic Obstructive Pulmonary Disease (COPD).

REFERENCES


ARs are located mainly in the cytoplasm (magnification 20 X) (A). However, in a few ASM cells ARs are also present in the cell nucleus (magnification 40 X) (B). The smooth muscle origin of cells was confirmed by immunofluorescence with the anti SM α-actin mouse monoclonal antibody A104 (1:400, Sigma) (magnification 20 X) (C). The position of the cell nucleus was visualized by DAPI. The fluorescent signal was analyzed with an Optiphoto-2 microscope and UFX-DX camera system (Nikon) at the indicated magnification.

Figure 2: Airway smooth muscle cells express classical Androgen Receptors (ARs). Airway smooth muscle cells (passages 3-6) were analyzed by immunofluorescence using an anti-human classical Androgen Receptor (AR) mouse monoclonal antibody (G122-25, 1:200, BD Transduction laboratories). ARs are located mainly in the cytoplasm (magnification 20 X) (A). However, in a few ASM cells ARs are also present in the cell nucleus (magnification 40 X) (B). The smooth muscle origin of cells was confirmed by immunofluorescence with the anti SM α-actin mouse monoclonal antibody A104 (1:400, Sigma) (magnification 20 X) (C). The position of the cell nucleus was visualized by DAPI. The fluorescent signal was analyzed with an Optiphoto-2 microscope and UFX-DX camera system (Nikon) at the indicated magnification.

There are a few studies investigating the impact of airway size on the modulatory role of airway epithelium. Studies on 2nd and 3rd order of canine airways revealed that epithelium mainly modulates the responsiveness of the large airways. On the contrary, we demonstrated that on sheep airways, epithelium integrity affects mainly the responsiveness of small airways. Specifically, acetylcholine or KCl-induced contraction of epithelium intact airways is independent of airway size. In contrast, the mechanical removal of epithelium affects mainly the responsiveness of 3rd and 4th order airways to acetylcholine. This difference seems to be attributed to the capability of epithelial cells to produce NO along the tracheo-bronchial tree.

AIRWAY SIZE

Several studies demonstrated that the responsiveness of smooth muscle to contractile agents varies in different parts of the bronchial tree and is influenced by airway innervation, receptor density, mechanical properties, airway wall anatomy and the distribution of acetylcholinesterase. In addition, structural and functional regional differences in airway epithelium have also been described. Regional differences in airway smooth muscle contractility are of physiological importance because smooth muscle contraction in central airways determines the resistance to airflow and gas distribution, whereas in peripheral airways it mainly controls the regional ratio of perfusion to ventilation. The regional differences in smooth muscle responsiveness to contractile agents may also be the basis of the non-homogeneous distribution of bronchoconstriction observed in pathological conditions such as Chronic Obstructive Pulmonary Disease (COPD) and asthma.

There are a few studies investigating the impact of airway size on the modulatory role of airway epithelium. Studies on 2nd and 3rd order of canine airways revealed that epithelium mainly modulates the responsiveness of the large airways. On the contrary, we demonstrated that on sheep airways, epithelium integrity affects mainly the responsiveness of small airways. Specifically, acetylcholine or KCl-induced contraction of epithelium intact airways is independent of airway size. In contrast, the mechanical removal of epithelium affects mainly the responsiveness of 3rd and 4th order airways to acetylcholine. This difference seems to be attributed to the capability of epithelial cells to produce NO along the tracheo-bronchial tree.

LOAD

During the tidal action of breathing load fluctuations are imposed continuously on ASM that undergo shortening and lengthening. Stress and strain can both be the mechanical signals involved in mechanosensitive modulation of ASM activation depending on the applied contractile stimuli. The mechanisms involved comprise ASM stiffness and extensibility, alterations in intracellular Ca²⁺ concentration and regulation of molecules involved in contractile protein activation. Despite the above involved mechanisms, the epithelium may also have a modulatory role in the mechanosensitive modulation of ASM responsiveness. To be precise, studies suggest that airway epithelium modulates ASM responsiveness to acetylcholine at increased load. This effect is mediated at least in part via NO release from epithelial cells. The involved pathway comprises the calcium dependent activation of epithelial NO synthase. As epithelium responds to stretch by modulating epithelial NO synthase activity, and thus NO production with a consequent reduction of airway responsiveness, this protective mechanism could be impaired in epithelial damage seen in airways diseases in particular asthma.


50. Pang JJ, Xu XB, Li HF, Zhang XY, Zheng TZ, Qu SY. Inhibition


82. Scichilone N, Permutt S, Togias A. The lack of the bronchoprotective and not the bronchodilatory ability of deep inspiration is associated with airway hyperresponsiveness. *Am J Respir Crit Care Med*. 2001; 163(2): 413-419. doi: 10.1164/ajrccm.163.2.2003119

Renal Salt Wasting Syndrome due to Carboplatin in a Patient with Lung Cancer


Department of Respiratory Medicine, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan

*Corresponding author:
Takeshi Saraya, MD, PhD
Assistant Professor
Department of Respiratory Medicine
Kyorin University School of Medicine
6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan
Tel/Fax: +81 (0) 422 44 0671
E-mail: sara@vd5.so-net.ne.jp

ABSTRACT

A 40-year-old woman with lung cancer had multiple episodes of hyponatremia whenever she had chemotherapy with cisplatin plus etoposide and/or carboplatin plus etoposide over the last year. Although she had been diagnosed as having Syndrome of Inappropriate Secretion of Antidiuretic Hormone (SIADH), based on a multidisciplinary assessment, a diagnosis of Renal Salt Wasting Syndrome (RSWS) possibly due to carboplatin was made, and after completion of intravenous treatment with isotonic saline, hyponatremia resolved, and she was discharged uneventfully. Cisplatin is well known for causing renal toxicity via proximal tubular damage, some cases of which present as RSWS. However, RSWS is extremely rare with carboplatin. The differential diagnosis between RSWS and SIADH for hyponatremic patients is sometimes difficult because of similarities in their clinical features, but careful consideration is needed to make the correct diagnosis because their treatments are diametrically opposed.

KEYWORDS: Renal salt wasting syndrome; SIADH; Hyponatremia; Carboplatin.

BACKGROUND

Hyponatremia has a large differential diagnosis and is a critical issue in cancer patients. The Syndrome of Inappropriate Secretion of Antidiuretic Hormone (SIADH) is a well-known cause of hyponatremia, while physicians generally overlook Renal Salt Wasting Syndrome (RSWS). This reflects the difficulty of differentiating between SIADH and RSWS based on limited clinical or laboratory findings. This case illustrates a useful way of discriminating between these two disorders and also shows that carboplatin is an extremely rare cause of RSWS.

CASE PRESENTATION

A 40-year-old woman was admitted to our hospital for chemotherapy. Two years ago she was diagnosed with small cell lung cancer (cT4N3M1a, stage IV, extensive stage) and was treated with 5 cycles of cisplatin (CDDP) plus etoposide (VP-16), followed by two cycles of carboplatin (CBDCA) plus VP-16. The latest chemotherapy was performed three weeks prior to current admission, and the lung cancer had been assessed as stable disease based on the evaluation criteria of solid tumours (RECIST) guidelines. However, she had multiple episodes of hyponatremia (minimum serum sodium: 116 mEq/L) whenever she received chemotherapy for the preceding year (total of five episodes). She was presumptively diagnosed with SIADH, and she improved with restriction of water intake in addition to supplementation of sodium levels at each time. At her current admission, the vital signs were normal, and physical examination showed a moderate pleural effusion in her right hemithorax. Laboratory examinations were
normal except for moderate anemia (8.2 g/dL) and leukocytopenia (1.9x10^3/μL). Her serum sodium was also normal (138 mEq/L) with oral intake of sodium (6 g/day). At this point, she did not satisfy the criteria for SIADH. Therefore, to investigate the differential diagnosis of the hyponatremia expected in due course with chemotherapy, an intensive survey for hyponatremia was performed at the day of admission using a 24-hour urine collection. From days 3 to 5, she was treated with intravenous CBDCA plus VP-16. On day 6, she had nausea and arthralgia and the hyponatremia (130 mEq/L) emerged together with hypouricemia (2.9 mg/dL) and increased fractional excretion of urate (FEna) (13%>10%) (Figure 1). However, on the same day, thyroid function (free T4: 1.23 ng/dL, free T3: 2.99 pg/mL, and thyroid stimulating hormone: 31.8pg/mL), serum cortisol (6.8 mg/dL), aldosterone (48.5 pg/mL), renin activity (0.4 ng/mL/hr), and brain natriuretic peptide (74.8 pg/mL) were all within normal limits. The 24-hour urine collection showed that urine osmolality (429 mOsm/kg) was higher than serum osmolality (263 mOsm/kg), and urine sodium (97 mmol/L) and chloride (87 mmol/L) levels were also high. No elevation of tubular enzymes were noted.

During the clinical course, presence of decreased Extracellular Volume (ECV) was assessed by: 1) decreased skin turgor; 2) dry axilla; 3) delayed capillary refill time (>2 sec); 4) dry mouth; 5) postural hypotension; and 6) jugular venous pressure. None of these were recognized from day 0 to day 10, so there was no severe depletion of ECV was noted throughout this phase. FENa (fractional excretion of sodium)>1% was seen from admission to the day 10 (day 0, 6, 8, and 10).

Based on the findings of hypouricemia (<4 mg/dL), increased FEna (>10%), and high urine sodium (>40 mmol/L) in addition to hyponatremia, RSWS was suspected due to CBDCA or SIADH. Table 1 compares expected laboratory values for RSWS and SIADH in hyponatremic patients, with the red letters consistent with the present case on day 6. After initiation of volume repletion therapy with isotonic saline (1 L/day for two days; day 6 and 7), the hyponatremia improved rapidly to 140 mEq/L over two days. This indicated that the hyponatremia was caused by RSWS, but not by SIADH.

Table 1: Differentiation of SIADH from RSWS.

<table>
<thead>
<tr>
<th></th>
<th>RSWS</th>
<th>SIADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postural hypotension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma renin</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>(normal, 2.8-8.7 μL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma aldosterone</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>(normal, 10-160 μg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum urate</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>FE urine (normal 5-20%)</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>FE phosphate (normal &lt;20%)</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>UNa</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>Serum Na after treatment with isotonic saline</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>ADH</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>ANP/BNP</td>
<td>N↑</td>
<td>N↑</td>
</tr>
<tr>
<td>Treatment</td>
<td>Salt loading/</td>
<td>Free water restriction/</td>
</tr>
<tr>
<td></td>
<td>volume replacement</td>
<td>hypertonic saline infusion</td>
</tr>
</tbody>
</table>

Figure 1: The clinical course of the present case.

**DISCUSSION**

Small cell lung cancer is a disseminated disease in most patients and the primary therapeutic modality is systemic chemotherapy for extensive stage disease as in the present case. The standard regimens are platinum-based combinations (CDDP plus VP-16 or CBDCA plus VP-16) because of their activity and toxicity profile. Carboplatin (CBDCA) has less nephrotoxicity and ototoxicity than cisplatin, with greater or comparable antitumor activity. However, sporadic observations of renal function deterioration after multiple courses of CBDCA have been reported, with reductions in the glomerular filtration rate or effective renal plasma flow. During the management of cancer patients, hyponatremic status needs to be carefully considered because it can be caused by diverse disorders, as well as concomitant use of other drugs acting on the serum sodium level. Among these disorders, differentiating between SIADH and RSWS is a critical issue because of their opposite treatments (i.e. fluid restriction done for management of SIADH can worsen the hyponatremia in RSWS, whereas fluid replacement with isotonic saline done for management of RSWS can worsen the hyponatremia in SIADH). From this perspective, the hyponatremia of the present case showed immediate correction via intravenous infusion of isotonic saline.
To the best of our knowledge, only two cases of RSWS associated with CBDCA have been reported with or without pulmonary damage due to either the antecedent 5 cycles of chemotherapy or the latest chemotherapy (3 weeks earlier) with CBDCA plus VP-16. Most previous cases of RSWS due to CDDP occurred from a few days to 10 days after completion of the chemotherapy and renal function recovered within the next three weeks. Thus, the present case had mild impaired renal dysfunction on admission (FEurate>10%), which would be derived from tubular depletion in RSWS, especially in mild RSWS with normal to slightly depleted ECV, as in the present case.

The elevated plasma renin, aldosterone, and ADH and low normal atrial natriuretic peptide levels are all physiologic consequences of RSWS. However, the extent of Extracellular Volume (ECV) depletion in RSWS seems to depend on the severity of renal dysfunction of sodium reabsorption. Furthermore, physical assessment of ECV is limited, which leads to a therapeutic dilemma: to restrict water for SIADH or to administer isotonic saline in RSWS, especially in mild RSWS with normal to slightly depleted ECV, as in the present case.

Previous studies showed that no increase in tubular enzymes (β2-microglobulin or N-acetyl-β-glucosaminidase (NAG)) was found in patients receiving CBDCA up to 520mg/m² but data on patients who developed CBDCA nephrotoxicity is unavailable. Santana et al. reported that 40% of patients receiving high-dose CBDCA and VP-16 with autologous marrow transplantation with relapsed solid tumours had hyponatraemia, but no reason was given.

The present case had mild impaired renal dysfunction on admission (FEurate>10%), which would be derived from tubular damage due to either the antecedent 5 cycles of chemotherapy with CDDP plus VP-16 (5th cycle was completed 3 months earlier) or the latest chemotherapy (3 weeks earlier) with CBDCA plus VP-16. Most previous cases of RSWS due to CDDP occurred from a few days to 10 days after completion of the chemotherapy and renal function recovered within the next three weeks. Thus, in the present case, the CBDCA seemed to be attributed to renal damage, and the renal dysfunction recovered at day 10 along with normalized FEurate or serum sodium. Sleijfer et al. reported that cumulative dose of CBDCA cause considerable loss of renal function with no increase in tubular enzymes or changes in the relative β2-microglobulin clearances, which might be have occurred in the present case.

This case reminds us of the importance of the concept of “RSWS”, which is difficult to diagnose but treatable.

CONFLICTS OF INTEREST: None

REFERENCES


Alpha-1 Antitrypsin Gene Polymorphism in the Egyptian Population: Association with Obstructive Lung Diseases

Rasha Daabis, Shaden Muawia, Amal Ahmed, Mohamed El-Shahat, Ahmed Youssef and Tarek Fekry

1Associate Professor of Chest Diseases, Department of Chest Diseases, Faculty of Medicine, Alexandria University, Egypt
2Professor of Chest Diseases, Department of Chest Diseases, Faculty of Medicine, Alexandria University, Egypt
2Professor of Biochemistry and Molecular Biology, Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt
2Professor and Head of Molecular Biology Department, Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt
2Professor of Biochemistry and Molecular Biology, Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt
2Assistant Lecturer of Molecular Biology, Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt

**ABSTRACT**

**Background:** Given the potential adverse effects of asthma and Chronic Obstructive Pulmonary Disease (COPD), this study was undertaken to explore Alpha-1 Antitrypsin (AAT) polymorphism in the Egyptian population and its role in the development and/or progression of asthma and COPD. The identification of IL-10 as a potential modifier gene for COPD susceptibility provided insight into additional inflammatory pathways to consider in AAT deficiency.

**Methods:** This study was carried on 90 unrelated Egyptians; 37 asthmatics, 33 COPD patients and 20 controls. Patients were evaluated clinically and with spirometry. The frequency of AAT gene polymorphism was assessed by real-time PCR. Serum levels of AAT protein, IL-10 and IgE were estimated.

**Results:** PiZ allele was found in COPD and asthma patients as well as controls. While PiS allele was never shown up in all the groups. The prevalence of PiZ was higher in asthma and COPD than in controls (75.75%, 72.7% and 50% respectively). Serum AAT was significantly decreased in patients with asthma and COPD. Patients with the PiZ allele, despite having lower values of the serum AAT, this difference was not significant. Serum AAT was significantly correlated with severity of airflow obstruction in both asthma and COPD. There was a significant elevation of serum IgE in COPD patients carrying PiZ allele. Serum IL-10 was significantly higher in asthma and COPD patients than the controls. There was a positive significant correlation between IL-10 and IgE in COPD patients.

**Conclusion:** The z allele frequency in the Egyptian population is higher among asthmatic and COPD patients, suggesting that it could in fact be an underlying hidden risk factor for the development of these diseases. Asthmatics carrying this deficient allele have a genetic predisposition for progressing to COPD. Genetic counselling of patients having obstructive airway diseases is very important for diagnosis, prognosis and treatment.

**KEYWORDS:** Alpha-1 antitrypsin deficiency; AAT gene polymorphism; Heterozygous PiMZ; Asthma; COPD; IL-10; IgE; Obstructive lung diseases.
ABBREVIATIONS: AAT: Alpha 1 antitrypsin; PI: Proteinase Inhibitor; COPD: Chronic Obstructive Pulmonary Disease; NE: Neutrophil Elastases.

INTRODUCTION

Alpha 1 antitrypsin (AAT) deficiency is a hereditary autosomal disorder, resulting from a variety of mutations in the alpha1-AT gene and associated with a high risk for the development of early-onset pulmonary emphysema. AAT is a highly polymorphic protein with more than 70 variants, known as Proteinase Inhibitor (PI) types. The Pi M allele and its serum subtypes are the most common of the normal alleles. The Pi Z is the commonest allele for the homozygous (PiZZ) severe deficiency that significantly increases the susceptibility to lung function loss and emphysema in smokers and non-smokers. PiMZ, the heterozygous condition, carries only a slightly higher independent risk of obstructive lung disease. The inheritance of an intermediate deficiency state such as PiSZ leads to intermediate susceptibility. AAT has a function of protecting the pulmonary parenchyma from the effects of Neutrophil Elastastases (NE) which are potent destructive proteases. In case of AAT deficiency, a gradual destruction of the pulmonary tissue occurs, resulting finally into Chronic Obstructive Pulmonary Disease (COPD), emphysema and early death. Along with the enhanced susceptibility to the development of Chronic Obstructive Pulmonary Disease (COPD) there may also be an enhanced susceptibility to asthma. Asthma is the most common respiratory diagnosis in patients with AAT Deficiency (AATD) prior to the diagnosis of AATD.

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are the most common obstructive lung diseases. They are both characterized by airway remodelling and chronic inflammation. Genetic factors play an important role in the development of these diseases, which has prompted much research to identify the underlying disease susceptibility genes. Given the potential adverse effects of asthma and COPD, this study was undertaken to explore AAT polymorphism in the Egyptian population and to elucidate the possible role of the Pi S and PiZ AAT alleles in the development and/or progression of asthma and chronic obstructive pulmonary disease. The identification of IL-10 as a potential modifier gene for chronic obstructive pulmonary disease susceptibility provided insight into additional inflammatory pathways to consider in alpha1-antitrypsin deficiency. Therefore, we estimated the serum level of AAT protein, IL-10 and IgE in our studied groups.

SUBJECTS AND METHODS

This study was conducted on 90 unrelated Egyptian persons, who were divided into three groups; group 1 included 37 asthmatic patients, group 2 included 33 COPD patients, and group 3 included 20 normal subjects as control. Asthmatic patients were diagnosed according to the standard clinical presentation and spirometry. Asthmatic patients exhibited a positive airway reversibility test, as defined by a post bronchodilator improvement of Forced Expiratory Volume in one second (FEV1) by more than 12% and 200 ml.

The diagnosis of COPD was based on the definition provided by the Global initiative for chronic Obstructive Lung Disease (GOLD), which is characterized by a post-bronchodilator Forced Expiratory Volume in one second (FEV1) to Forced Vital Capacity (FVC) ratio of <70% and a post bronchodilator reversibility of <12% and 200 ml.

The control group included normal subjects who were recruited from the general population and had no respiratory symptoms, and no evidence of airflow obstruction. They were excluded if they had a history of atopy, an acute pulmonary infection in the 4 weeks preceding assessment for the study, or a family history of asthma or COPD. All the cases and controls were unrelated Egyptian people who were selected from the same population. The cases were recruited from the inpatient and the outpatient clinic of the chest diseases department, Alexandria main university hospital. All subjects were enrolled in the study after a written informed consent according to the protocol approved by the Ethics Committee of the Alexandria Main University Hospital.

Quantitative determination of Immunoglobulin E

Measurement of Immunoglobulin E (IgE) by The Electrochemi-luminescence immunoassay (ECLIA) technique based on the sandwich principle. It was used on Elecsys and (cobas e) immunoassay analyzers (Roche Diagnostics GmbH, Mannheim, Germany). Using the mean absorbance value for each sample determine corresponding concentration of Total IgE in IU/ml from the standard curve.

Quantitative determination of AAT level

Measurement of Alpha 1 antitrypsin (AAT) serum level by Radial Immune Diffusion (RID) plates (BIOCIENTIFICA S.A., Buenos Aires-Argentina).

Quantitative determination of serum level of Interleukin-10

Measurement of plasma IL-10 was done by Enzyme-Linked Immunosorbent Assay (ELISA) method. Avi Bion-Human IL-10 ELISA Kit was used (Orgenium Laboratories-Vantaa, Finland).

Genotyping

Genomic DNA was isolated from 300 μL of whole venous blood using QIAamp DNA Mini and Blood Mini...
kit (QIAGEN, HILDE, GERMANY). Real-time PCR mutation detection by allelic discrimination snpsig kit (Primer Design® Ltd), (Applied Biosystems Corporation) - California, USA. After optimizing the thermal cycling conditions, the reaction plate was loaded into the thermal cycler (Rotor–Gene Q, Applied Biosystems). The genotype of each sample is calculated by comparing the ratio of signals between the two channels (ROX and VIC).

**STATISTICAL ANALYSIS**

Data were collected, tabulated, then analyzed using SPSS version 13. Qualitative data were presented as numbers and percentage. Quantitative data were described using mean and standard deviation. Comparison between different groups regarding categorical variables was tested using Chi-square test. For normally distributed data, comparison between COPD, asthma and control groups were done using F-test (ANOVA) and pair wise comparisons; between each two groups was assessed using Post Hoc test (Scheffe), while for abnormally distributed data comparisons between the three groups was done using Mann Whitney test. Also the comparisons between mutant and wild cases in COPD or asthmatic groups was done either with Student t-test or Mann Whitney according to the normality of data. Significance test results are quoted as two-tailed probabilities. For all statistical tests, a p-value of <0.05 was considered significant.

**RESULTS**

**Characteristics of the studied groups**

The characteristics of the three studied groups are presented in Table 1. There was a significant positive family history in asthmatic patients in comparison to the COPD group (p<0.001). However, the airway obstruction was more severe in the COPD group than the asthmatic patients as measured by the FEV₁/FVC (p=0.017).

**Comparison between the three studied groups according to serum IgE, AAT and IL-10 levels (table 2)**

Serum IgE was significantly elevated in the asthmatic group in comparison to the COPD and control groups (p ≤ 0.001 for both). Also it was elevated in the COPD group in comparison to the control group (p ≤ 0.01). (Table 5)

Serum AAT was significantly lower in the COPD and the asthmatic group in comparison to the control group at p ≤ 0.01 and ≤ 0.05 respectively.

IL-10 was significantly higher in the COPD and the asthmatic group in comparison to the control group at p ≤ 0.01 and ≤ 0.05 respectively.

**Correlation between different parameters in COPD group (table 3)**

In the COPD group, the serum AAT was positively correlated with FEV₁, FVC and FEV₁/FVC (p<0.001, 0.001 and 0.004 respectively). Concerning the IL-10, it was positively correlated with IgE (P<0.001).

**Correlation between different parameters in asthma group (table 4)**

In the Asthma group, the AAT revealed positive significant correlation with FEV₁, FVC and FEV₁/FVC (p =0.034, 0.031 and 0.014 respectively). Also, IL-10 showed positive significant correlation with FEV₁, FVC and FEV₁/FVC (P =0.020, 0.017 and 0.049 respectively).
Comparison between the three studied groups according to Z allele (PiZ) (table 5)

The M wild type allele was found in 9 COPD patients (27.3 %) while the PiZ mutant type allele was found in 24 COPD patients (72.3 %). The M wild type allele with Z primers was found in 9 asthma patients (24.3 %) while the PiZ mutant type allele was found in 28 Asthma patients (75.7%).

In the control group the percentage of wild and mutant types of PiZ allele was 50% for both types.

This variation among the three groups was not statistically significant. The only significant difference was observed between Asthma group and control group (P=0.049) concerning wild and mutant type of PiZ (Table 5).

Comparison between the patients’ group and the control group according to PiZ (table 6)

When grouping the COPD and asthmatic patients in a single group and comparing them to the control group we found that the patients’ group presented more with the mutant type of PiZ than the control group (p= 0.039).

Relationship of the PiZ allele with different clinical and laboratory parameters in COPD and asthma group (table 7)

Patients carrying the mutant PiZ allele in the asthma group didn’t reveal any significant difference in the clinical or laboratory parameters in comparison to patients with wild type. However, in the COPD group, we found that COPD patients with the PiZ mutant allele had a significantly higher serum IgE (P = 0.015) in comparison to patients with wild type.

DISCUSSION

Alpha-1 antitrypsin deficiency (AATD) is a hereditary recessive autosomal disease caused by mutations in the AAT gene. This disease is characterized by abnormally low AAT concentrations in plasma.16 The clinical manifestations of AATD...

<table>
<thead>
<tr>
<th>COPD (n=33)</th>
<th>Asthma (n=37)</th>
<th>Control (n=20)</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PiZ</td>
<td></td>
<td></td>
<td>4.352</td>
<td>0.114</td>
</tr>
<tr>
<td>Wild type (M)</td>
<td>9  27.3</td>
<td>9  24.3</td>
<td>10  50.0</td>
<td></td>
</tr>
<tr>
<td>Mutant type (PiZ)</td>
<td>24  72.7</td>
<td>28  75.7</td>
<td>10  50.0</td>
<td></td>
</tr>
<tr>
<td>P₁</td>
<td>0.140</td>
<td>0.049</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₂</td>
<td>0.778</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

χ²: Chi square test
p₁: p value for Chi square test for comparing between control and each patient group
p₂: p value for Chi square test for comparing between COPD and Asthma group
*: Statistically significant at p < 0.05
**: Statistically significant at p < 0.01
***: Statistically significant at p < 0.001

Table 5: Allele frequency in the three studied groups according to PiZ

Comparison between the patients’ group and the control group according to PiZ (table 6)

When grouping the COPD and asthmatic patients in a single group and comparing them to the control group we found that the patients’ group presented more with the mutant type of PiZ than the control group (p= 0.039).

<table>
<thead>
<tr>
<th>Cases (n=70)</th>
<th>Control (n=20)</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. %</td>
<td>No. %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PiZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (M)</td>
<td>18  25.7</td>
<td>10  50.0</td>
<td></td>
</tr>
<tr>
<td>Mutant type (PiZ)</td>
<td>52  74.3</td>
<td>10  50.0</td>
<td></td>
</tr>
</tbody>
</table>

χ²: Chi square test
p: Statistically significant at p < 0.05
**: Statistically significant at p < 0.01
***: Statistically significant at p < 0.001

Table 6: Comparison between the allele frequency in the patients and control group according to PiZ

Comparison between the three studied groups according to Z allele (PiZ) (table 5)

The M wild type allele was found in 9 COPD patients (27.3 %) while the PiZ mutant type allele was found in 24 COPD patients (72.3 %). The M wild type allele with Z primers was found in 9 asthma patients (24.3 %) while the PiZ mutant type allele was found in 28 Asthma patients (75.7%).

In the control group the percentage of wild and mutant types of PiZ allele was 50% for both types.

This variation among the three groups was not statistically significant. The only significant difference was observed between Asthma group and control group (P=0.049) concerning...
To our knowledge, there are no available data about the AAT deficiency status in our country. Also Edwin and Robert showed that, AAT deficiency remains undiagnosed in many patients, and there are often long delays between the onset of respiratory symptoms and diagnosis, and the condition is frequently not diagnosed. Therefore we sought to uncover any underlying mutations of the alpha1-AT gene in our population and their role in the predisposition to COPD or asthma.

The results of the study showed that PiZ allele was found in COPD and asthma patients as well as controls. While PiS allele was never shown up in all the groups. Moreover, the prevalence of PiZ was higher in asthma and COPD than in controls (75.75%, 72.7% and 50% respectively), suggesting that the presence of this deficiency allele could in fact be an underlying hidden factor that increased the susceptibility to the development of these diseases in our population.

Table 7: PiZ allele distribution according to IgE, AAT and IL-10 in each group of patients

<table>
<thead>
<tr>
<th>COPD</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M Wild</strong></td>
<td><strong>PiZ Mutant</strong></td>
</tr>
<tr>
<td>allele (n = 9)</td>
<td>allele (n = 24)</td>
</tr>
<tr>
<td>IgE</td>
<td></td>
</tr>
<tr>
<td>Mean±SD</td>
<td>35.63 ± 29.07</td>
</tr>
<tr>
<td>Z (p)</td>
<td>2.426* (0.015)</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>104.12 ± 26.48</td>
</tr>
<tr>
<td>Z (p)</td>
<td>1.497 (0.135)</td>
</tr>
<tr>
<td>IL-10</td>
<td>4.13 ± 1.97</td>
</tr>
<tr>
<td>Z (p)</td>
<td>0.101 (0.919)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AAT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>269.84 ± 256.16</td>
</tr>
<tr>
<td>Z (p)</td>
<td>0.015* (0.915)</td>
</tr>
</tbody>
</table>

Table 7: PiZ allele distribution according to IgE, AAT and IL-10 in each group of patients

We have observed a decreased serum level of AAT in patients with asthma and COPD these results are in agreement with other studies carried by various workers. However, patients with the PiZ allele, despite having lower values of the serum AAT than those with the wild type, this difference was not statistically significant, which might need a larger studied population to demonstrate a statistically significant difference. The AAT plasmatic concentration may vary in the patient’s sample with other physiological or pathological states like age, asthma duration, an acute bronchial inflammation and/or the corticoid treatments.

Moreover, some reported data suggest that besides the AAT deficiency, smoking, atopic constitution and other factors may also contribute to the progression of pulmonary lesions which might explain this lack of statistical significance due to the interplay of several factors.

We have found that the serum concentration of AAT was significantly correlated with the severity of airway obstruction in both asthmatic and COPD patients. In their study, Eden et al. suggested that individuals with AATD lack a major anti protease defence against airway inflammation; they are more susceptible to allergen-mediated asthma and consequent progressive airway obstruction. Such patients may be candidates for measures aimed at reducing the impact of environmental aeroallergens.

The monitoring of these patients with wide-range lung function variations should provide an additional insight into the origin and pathogenesis of obstructive lung diseases correlated to the AAT deficiency.
Asthma and COPD have long been considered to be separate disease entities due to their different clinical phenotypes. There are, however, similarities in the types of inflammatory cells observed in the airways of patients with these diseases, and cytokines secreted by these types of cell interact as a network of inflammatory mediators.\(^3\)

Considering the important role of cytokines in COPD and asthma, it is necessary to define the IL-10 as an inflammatory mediator. IL-10 has pleiotropic effects in immune regulation and inflammation. In addition, IL-10 has been known to inhibit the lymphokine production by Th1 but not Th2 clones and down regulate the Th1 cell differentiation.\(^2\)

The results of the present study showed that the levels of IL-10 in patients of Asthma and COPD are significantly higher than the control group (P ≤ 0.001). The elevated level of IL-10 in the serum of asthma subjects indicated an increase in Type-2 activity through which the production of IL-4 and IL-13 may promote an isotype switch to IgE. Thus, a prominent shift in patient’s cytokine milieu from Type-1 to Type-2 may have resulted in the elevated levels of total IgE which was also demonstrated in our asthma and COPD patients.

There have been conflicting reports in literature on the levels of IL-10 in asthma patients. Kumar et al., showed that the levels of IL-10 in patients of asthma increased significantly (P = 0.001) in comparison to controls, he also found an increase in a big panel of cytokines (IL-1β, IL4, IL5, IL6, IL8) and he postulated that; these observations emphasized the fact that there is a complex series of inflammatory events in this disorder where eosinophils and neutrophils play interactive roles.\(^2\)

However, Takaashi et al., in their study on Japanese subjects reported reduction in IL-10 level in sputum of bronchial asthma and in normal smokers as compared to healthy non-smokers.\(^3\) In a study carried out by Ceyhan et al., IL-10 in sera and induced sputum of asthma patients were found to be unaltered.\(^3\)

Moreover, a study was done by Bhadoria et al. on inflammatory cytokines in Indian COPD patients, the study was undertaken for a cytokine profile including IL-10 and other cytokines (IL-1β, IL-4, IL-5, IL-6, IL-8) which showed a marked significant increase in serum concentration of IL-10 and the other cytokines in COPD patients compared to healthy controls. This pattern of serum cytokines indicates a switch of type-1 to type-2 cytokine predominance that may result in enhanced synthesis of IgE creating a systemic inflammatory response.\(^3\) This is further supported by our finding of a positive significant correlation between IL-10 and IgE levels in COPD patients (P<0.001).

Also we found that the COPD patients carrying the mutant allele PiZ had a significantly higher levels of serum IgE (p=0.015) which might indicate that asthmatic patients carrying this deficient allele might have a genetic predisposition for progressing to COPD.

The development of asthma in patients with AATD may have additive long-term effects on the development of irreversible airway obstruction and emphysema. In this regard, both an increased serum IgE titer and atopy have been associated with the development of chronic obstructive lung disease.\(^2\)

An increased serum IgE level is also not specific for asthma. It is associated with cigarette smoking,\(^2\) and may be a marker of airway inflammation.\(^2\) However, it is unlikely that smoking was the causative factor in our study, since the proportion of current smokers in the COPD and the control groups did not show significant difference. In addition, there was no significant difference between smokers and non-smokers in mean total serum IgE concentrations. Therefore, airway inflammation or underlying asthma is a more likely cause of the increased mean serum IgE in COPD patients with the mutant allele PiZ.

Over the long term, asthma may have an adverse impact on lung function in persons with AATD. Chronic bronchial-wall inflammation could result in structural remodelling that leads to irreversible narrowing of airways. In this regard, Villar and co-workers reported that atopy and bronchial responsiveness in elderly, former and current smokers, predisposes to an accelerated decline in FEV\(_1\).\(^3\)

A significant proportion of patients with severe AATD and advanced emphysema show clinical features of asthma, and asthma appear to be more common in patients with this condition than in those with COPD and a normal Pi phenotype. The increased serum IgE level indicates that allergic mechanisms could contribute to the development of chronic airway obstruction. It is suggested that because individuals with AATD lack a major anti protease defence against airway inflammation, they are more susceptible to allergen-mediated asthma and consequent progressive airway obstruction.\(^3\) In addition, increasing the concentration of AAT in these patient’s airways might ameliorate the effect that environmental factors have on them.\(^3\)

In conclusion, this study demonstrated that the z allele frequency in the Egyptian population is higher among the asthmatic and COPD patients, suggesting that it could in fact be an underlying hidden risk factor for the development of these diseases. The early identification of this mutant allele and other polymorphisms presents predictive and therapeutic avenues in the context of obstructive airway diseases. Asthmatics carrying this deficient allele have a genetic predisposition for
progressing to COPD. Genetic counseling of patients having obstructive airway diseases is very important for diagnosis, prognosis and treatment.

DECLARATION OF INTEREST

I declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

FUNDING

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

REFERENCES


Microarray Analysis Identifies Pathways In Progression of Early Stage Lung Adenocarcinoma: The Importance of Focal Adhesion and ECM-Receptor Interactions

Susan E. Douglas 1#*, Drew C. Bethune 2# and Zhaolin Xu 3#

# equally contributed

1 National Research Council Halifax, 1411 Oxford Street, Halifax, NS, B3H 3Z1, Canada
2 Department of Surgery, Queen Elizabeth II Health Sciences Centre and Dalhousie University, 1278 Tower Rd, Halifax, NS, B3H 2Y9, Canada
3 Department of Pathology, Queen Elizabeth II Health Sciences Centre and Dalhousie University, 5788 University Ave., Halifax, NS, B3H 1V8, Canada

ABSTRACT

Recurrence after lung cancer surgery is high, even among Non-Small Cell Lung Cancer (NSCLC) adenocarcinoma patients diagnosed early as Stage I, where there has been no spread to lymph nodes. Understanding the biological underpinnings of aggressivity and recurrence in this subset of tumours may enable the identification of patients who would benefit from adjuvant therapy. The purpose of this study was to identify differentially expressed molecular biomarkers that might underlie recurrence of Stage I tumours by comparing gene expression in later-stage tumours with those expressed in early-stage tumours. Gene expression in tissue biopsy samples from five Stage I and five Stage II/III NSCLC adenocarcinoma patients was analysed using an oligonucleotide microarray containing 17,000 probes printed in duplicate. Analyses were performed on total RNA isolated from tumour tissue of each patient using universal human RNA as a reference. Compared to normal tissues, the transcriptome of Stage I NSCLC adenocarcinomas showed enrichment in general pathways in cancer, whereas in Stage II/III more specific cancer pathways such as focal adhesion and ECM-receptor interaction pathways were enriched and components of the PPAR signalling pathway were depleted. Relative to early-stage NSCLC, Stage II/III adenocarcinomas showed up-regulation of genes of the basic transcriptional and translational machinery, particularly the “cancer testis antigen” PASD1 transcription factor. The actin cytoskeleton re-organisation and interleukin-6 pathways were also up-regulated whereas there was a generalized down-regulation of immune effectors and genes involved in immune system development. This small-scale transcriptome study provides important information about the pathways and molecules likely to be involved in the more metastatic propensity of those Stage I NSCLC adenocarcinomas that recur.

KEYWORDS: Adenocarcinoma; Biomarkers; Microarray; Non-small cell lung cancer; Recurrence; Transcriptome.

ABBREVIATIONS: MMP: Matrix Metalloproteases; NSCLC: Non-Small Cell Lung Cancer; WG: Web Gestalt.
INTRODUCTION

Lung cancer remains the leading cause of cancer-related death worldwide accounting for over a quarter (27%) of all cancer deaths each year.1 Non-Small Cell Lung Cancer (NSCLC) comprises ~80% of all lung cancers, and the majority of patients are diagnosed at an advanced stage that is associated with a poor clinical outcome and low survival (<16% overall 5-year survival).1 Even for patients classified as Stage I, the rate of post-operative recurrence is high, with a 5-year survival of only 52%. Risk factors for completely resected Stage IA tumours include poor differentiation, vascular invasion, wedge resection and minimal margins.2 Existing methods of classification and staging such as Tumour, Node, Metastasis (TNM)3 have great prognostic utility; however, patients with tumours of identical histology, differentiation, location and stage classifications may differ widely in their survival time or response to therapy.4 Misclassification of Stage I tumours can occur when lymph node metastases are small and escape detection. There is a need to incorporate molecular profiling with standardized criteria from radiology and medical oncology into the classification of NSCLC.5 Characterization of the basic underlying molecular alterations that occur during progression of early-stage NSCLC is urgently needed.

Various approaches have been attempted to aid in the classification, diagnosis and prognosis of NSCLC, including assessment of cells and nucleic acids (DNA, mRNA and microRNA) in sputum, bronchial biopsies, bronchial washing and brushing specimens, bronchial lavage fluid, blood, pleural effusions and solid tumour biopsies.6 Several cellular tumour markers have shown potential in predicting survival in NSCLC.7 Positive immunohistochemical staining of mTOR has been proposed as a marker of poor outcome in early stage NSCLC.8 Apo lipoprotein E is over-expressed in lung adenocarcinomas with malignant pleural effusion and is associated with poorer survival in these patients.9 SOX2 is over-expressed in the subset of Stage I adenocarcinomas from patients with poorer outcome and may help predict recurrence.10

Circulating plasma nucleic acid is elevated in lung cancer patients relative to controls and when detected at the time of diagnosis has been shown to be prognostic for poorer survival.11 RT-PCR assays have detected KRT19 and TTF-1 mRNA12 and TRIM2813 transcripts in peripheral blood of NSCLC patients, indicative of circulating tumour cells. However, circulating nucleic acid markers lack organ specificity, have poor sensitivity and there is often overlap between markers originating from benign and malignant tumours.

Transcriptome analyses have proved useful in the diagnosis and prognosis of NSCLC. The transcriptomic signature differs between malignant, normal and lung metastatic tumours of non-pulmonary origin. In addition, transcriptomics can be used to distinguish between different histological subtypes and stages of lung cancer, to improve prediction of clinical outcome and response to therapy.14 However, recent reviews of transcriptomic studies found that there was little consensus when it came to prognostic signatures.2,14 although a meta-analysis of seven different microarray studies did reveal a set of 64 genes whose expression was associated with survival of Stage I NSCLC patients.15 Most biomarker studies in NSCLC comprise patient cohorts including all stages and/or all histological subtypes of lung cancer;16 there are few microarray studies only comparing patients with early stage adenocarcinoma.17,18 One recent study compared three Stage IA and thirteen Stage IB NSCLC samples and found there was only one down-regulated gene (DSG3; desmoglein3) that discriminated between them.19

Biomarkers that predict metastatic potential of NSCLC at an early stage could significantly improve survival by identifying patients at high risk for recurrence and/or metastasis who may benefit from adjuvant chemotherapy.20 Since the pattern of gene expression in higher stage tumours can be informative for predicting risk of recurrence in Stage I tumours,21 we performed a pilot study that compared the transcriptomic profiles of tumours from patients with Stage I and Stage II/III adenocarcinoma and related these findings to the biological processes that may be involved in early recurrence/metastasis.

MATERIALS AND METHODS

Study population

Patient biopsy samples from five Stage I (with no lymph node involvement) and five Stage II/III (with lymph node involvement) patients were selected from the Queen Elizabeth II Health Sciences Center Lung Tumour Bank based on the following criteria: adenocarcinoma, age >45 years; all but one were current or past smokers (Table 1).

<table>
<thead>
<tr>
<th>ID</th>
<th>Stage</th>
<th>Age</th>
<th>Gender</th>
<th>Smoking Status</th>
<th>Recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L202</td>
<td>IB</td>
<td>76</td>
<td>Male</td>
<td>Current</td>
<td>no</td>
</tr>
<tr>
<td>L218</td>
<td>IA</td>
<td>65</td>
<td>Male</td>
<td>Past</td>
<td>yes</td>
</tr>
<tr>
<td>L252</td>
<td>IA</td>
<td>81</td>
<td>Male</td>
<td>Past</td>
<td>no</td>
</tr>
<tr>
<td>L272</td>
<td>IA</td>
<td>79</td>
<td>Female</td>
<td>Past</td>
<td>no</td>
</tr>
<tr>
<td>L278</td>
<td>IA</td>
<td>69</td>
<td>Male</td>
<td>Past</td>
<td>no</td>
</tr>
<tr>
<td>L229</td>
<td>IIA</td>
<td>66</td>
<td>Male</td>
<td>Past</td>
<td>yes</td>
</tr>
<tr>
<td>L240</td>
<td>IIA</td>
<td>74</td>
<td>Male</td>
<td>Past</td>
<td>no</td>
</tr>
<tr>
<td>L258</td>
<td>IIA</td>
<td>45</td>
<td>Male</td>
<td>Never</td>
<td>no</td>
</tr>
<tr>
<td>L262</td>
<td>IIA</td>
<td>76</td>
<td>Female</td>
<td>Past</td>
<td>no</td>
</tr>
<tr>
<td>L300</td>
<td>IIB</td>
<td>54</td>
<td>Female</td>
<td>Past</td>
<td>no</td>
</tr>
</tbody>
</table>

Table 1: Patient characteristics and samples used for transcriptome analysis.

This study was approved by the Capital Health Research Ethics Board (CDHA-RS/2004-336), and all participating individuals signed informed consent.
RNA extraction

Frozen lung tissue (50 mg) was pulverized in a Multi Sample Bio Pulverizer (BioSpec) and homogenized in 1 mL TRIZOL® (Life Technologies) using a FastPrep®24 (MP Biomedical). Total RNA was extracted according to the manufacturer’s protocol, treated with TURBO DNase (Applied Biosystems), purified with the Total RNA Purification Kit (Norgen Biotech Corp.) and quantified on a NanoDrop-1000 (Nano Drop Products).

Microarray analysis

Equal amounts (1 μg) of Universal Human RNA (Invitrogen) and lung tumor RNA samples were converted to oligo-modified cDNA using the 3DNA Array 900 Kit (Genisphere). cDNA samples were co-hybridized at 56 °C for 16 h on an oligonucleotide microarray containing 17,000 probes printed in duplicate (Atlantic Cancer Research Institute, Moncton, NB). A second hybridization was performed to bind the 3DNA Capture Regents, at 56 °C for 4 h. After washing, microarrays were scanned in an Axon GenePix 4200A scanner (Molecular Devices), gridded using GenePix software (version 6.0), and the .gpr output files were loaded into the ArrayPipe server at the National Research Council Halifax. Control spots and low quality spots were flagged, the remaining spots were background-corrected using the limma normexp option with a cutoff of 50, intensity data were normalized using the limma loess sub grid option, and duplicate spot data were merged. Spots that had a normalized corrected intensity value of >100 in either channel 1 or channel 2 were analysed using Limma’s empirical Bayes moderated t-test to identify spots that were significantly (p<0.05) differentially expressed between tumour and control reference RNA. Empirical Bayesian methods are used to provide stable results even when the number of arrays is small23 as in this study. The Student’s t-test module of MeV24 was then used to identify spots that were significantly differentially expressed between the Stage I and Stage II/III samples. Gene enrichment analysis to determine signalling pathways and biological processes involved in early stage NSCLC progression was performed of genes that were greater than two-fold differentially expressed using Web Gestalt (http://bioinfo.vanderbilt.edu/webgestalt) and DAVID (http://david.abcc.ncifcrf.gov/).

RESULTS AND DISCUSSION

In order to minimize effects of individual genetic differences and potential confounding signals that can arise from residual cancer cells in the normal tissue surrounding a tumours, 25-26 RNAs from tumour samples were compared to control universal RNA rather than adjacent normal tissue and then differences in these expression ratios were compared between stages. After background correction, normalization and filtering, 16752 genes could be compared in the microarrays from Stage I and Stage II/III NSCLC tumours. The data are available in GEO under the accession number GSE28956.

In Stage I tumours, 647 genes were up-regulated and 711 genes were down-regulated and in Stage II/III tumours, 868 genes were up-regulated and 1051 genes were down-regulated more than two-fold relative to the control universal reference RNA (p<0.05; Supplemental Tables 1 and 2).

Genes differentially expressed in Stage I and Stage II/III NSCLC adenocarcinomas

Cytkeratins are valuable markers of lung cancer and have long been used to differentiate between different sub-types of this cancer; more recently they have shown promise as prognostic markers in lung cancer. They are major components of the cytoskeletal system and play a role in cell migration, invasion and metastasis. As expected, cytokeratins 7, 8, 16 and 19 (typical markers of NSCLC) were up-regulated in both stages relative to control universal reference RNA (Table 2; Supplemental Tables 1 and 2).

Interestingly, KRT19 was expressed almost two-fold higher in Stage II/III compared to Stage I NSCLC tumours. Consistent with our results, high serum concentrations of KRT19 fragments (CYFRA 21-1) are prognostic of poor outcome in adenocarcinoma and tumours from patients with high pre-operative CYFRA 21-1 are larger and more poorly differentiated, indicative of a more aggressive nature.27

Cellular adhesion molecules (CEACAM1, 5, 6, 7, 8) and extracellular matrix proteins (LAMB3, LY6D, OLFM4, PSG1, SFN, VCAN, COL1A1, NAPSB, PKP) were also highly expressed in tumours relative to normal. Adhesion pathways have long been used to differentiate between different sub-types of NSCLC, particularly Stage I adenocarcinoma, and its prognosis was confirmed by immunohistochemistry in tissue microarrays.30 In our study CADM1 showed 3.2-fold higher expression in Stage I tumours than normal (Supplemental Table 1). CEACAMs are important regulators of invasion and metastasis and CEACAM6 inhibits cell-cell contact inhibition mediated by CEACAM1 in A549 lung adenocarcinoma cells,31 inducing cellular proliferation.32 CEACAM6 was up-regulated 37-fold in Stage I and 9-fold in Stage II/III tumours; interestingly, both CEACAM6 and surfactant proteins that it interacts with are targets of transcription factor TTF-133 and all are up-regulated.

The putative oncogene RHEB34 plays a role in growth and cell cycle progression through the AKT/MTOR pathway; it was up-regulated over 14-fold in Stage II/III tumours. Induction of angiogenesis, one of the hallmarks of cancer, is a major contributor to solid tumour development. CHI3L1, which
promotes tumour angiogenesis and has been shown to be prognostic for low survival in NSCLC, was up-regulated more than 10-fold in Stage II/III tumours and 6-fold in Stage I tumours. Several galectins (LGALS), which are also implicated in tumour angiogenesis as well as progression, were up-regulated in tumours of both stages.

The S100 family are calcium-binding proteins with varied roles in cancer invasion, metastasis and recurrence. They regulate a host of intracellular processes including enzyme activities, components of cytoskeleton, motility and cell cycle. An earlier microarray study showed calcium-binding proteins S100P and S100A2, and trypsinogens TRY6 and PRSS3 to be correlated with progression to metastasis in NSCLC. In our study, expression of S100P was more than 8-fold higher in both Stage I and Stage II/III tumours but S100A2 was up-regulated only in Stage II/III tumours (5.3-fold). Other S100 proteins such as S100A10, which is essential for migration of tumour-promoting macrophages into tumor sites, showed increased gene expression in Stage II/III tumours. Although TRY6 and PRSS3 trypsinogens were not differentially regulated in our study, matrix metalloproteases (MMPs) were dramatically up-regulated in Stage I and Stage II/III tumours, particularly the latter. MMPs also play a crucial role in metastasis by degrading extracellular matrix thereby allowing cell migration. Proteomics and immunohistochemical staining have shown that members of the annexin family (ANX) promote cancer cell invasion and metastasis in cancer, particularly ANXA1, ANXA2, ANXA4 and ANXA5. We found expression of ANXA1, ANXA2, and ANXA3 was higher in tumour tissue than normal.

Many of the up-regulated genes in the Table 2 such as LY6D, GZMB, IL7R, CXCL9, CXCL17, MZB1, and LGALS4, may be derived from tumour-infiltrating immune cells. Such immune genes may be prognostic in NSCLC, and IL7R and CXCR4 were identified as key players in the tumour microenvironment by gene profiling. In our study, CXCR4 showed a 2.8-fold increase in gene expression in Stage II/III tumours compared to Stage I. Interestingly, several of the genes were also included in a list of the top 20 up-regulated genes in the lung cancer

| Table 2: Top 25 up-regulated genes in Stage I and Stage II/III NSCLC adenocarcinomas relative to control RNA (identified using Limma’s empirical Bayes moderated t-test at p<0.05). |
|--------------------------------------------------|------------------|------------------|
| Gene                                             | Ratio            | Gene             | Ratio            |
| Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) | 37.65            | Pregnancy specific beta-1-glycoprotein 1 (PSG1) | 47.04            |
| Stratifin (SFN)                                  | 25.71            | Matrix metalloprotease 1 (MMP1)                 | 19.64            |
| Cytokeratin 16 (KRT16)                           | 21.75            | Cytokeratin 16 (KRT16)                           | 17.35            |
| Prostaglandin-endoperoxide synthase 2 (PTGS2- COX2) | 21.42            | Cytokeratin 19 (KRT19)                           | 16.12            |
| Olfactomedin 4 (OLFM4)                           | 18.18            | Ras homolog enriched in brain (RHEB)            | 14.46            |
| Solute carrier family 6, member 14SLC6A14        | 16.81            | FOS-like antigen 1 (FOSL1)                      | 13.68            |
| Lymphocyte antigen 6 (LY6D)                      | 15.20            | Steroid 5 alpha-reductase 2-like (SRD5A3)       | 13.45            |
| Seizure related 6 (SEZ6L)                        | 14.54            | Marginal Zone B And B1 Cell-Specific (MZB1)     | 12.83            |
| Serine protease inhibitor, clade B, member 5 (SERPINB5) | 14.46            | Serine protease inhibitor, clade B, member 5 (SERPINB5) | 12.45 |
| Granzyme B (GZMB)                                | 11.78            | Galectin 4 (LGALS4)                             | 12.29            |
| Pregnancy specific beta-1-glycoprotein 1 (PSG1)  | 10.50            | Stratifin (SFN)                                 | 11.78            |
| Laminin B3 (LAMB3)                               | 10.49            | Chemokine (C-X-C ligand 17 (CXCL17)             | 11.42            |
| Cellular retinoic acid binding protein 2 (CRABP2) | 10.06            | Oncostatin M receptor (OSMR)                    | 10.34            |
| Cytokeratin 8 (KRT8)                             | 9.85             | Chitinase-like 3 (CHI3L1; YKL40)                 | 10.17            |
| Cytokeratin 7 (KRT7)                             | 9.50             | TNF alpha-induced protein 2 (TNFIP2)            | 9.77             |
| Ligand dependent nuclear receptor corepressor (LCOR) | 9.47             | Neuroumedin U (NMU)                             | 9.71             |
| Pleckstrin homology domain interacting protein (PHIP) | 9.38             | Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) | 9.44 |
| Forkhead box P1 (FOXP1)                          | 9.07             | Versican (VCAN)                                 | 9.36             |
| Interleukin 7 receptor (IL7R)                    | 8.67             | Collagen, type I, alpha 1 (COL1A1)              | 9.25             |
| Solute carrier family 12, member 2 (SLC12A2)     | 8.63             | Cellular retinoic acid binding protein 2 (CRABP2) | 9.24 |
| Cytokeratin 19 (KRT19)                           | 8.61             | Laminin B3 (LAMB3)                              | 9.19             |
| CEP57L                                          | 8.44             | Napsin B (NAPSB)                                | 9.17             |
| S100 Ca++ binding protein P (S100P)              | 8.43             | S100 Ca++ binding protein P (S100P)             | 8.89             |
| Glutathione peroxidase 2 (GPX2)                  | 8.02             | X (inactive)-specific transcript (XIST)         | 8.71             |
| Chemokine (C-X-C ligand 9 (CXCL9)                | 7.96             | Piokpholin 3 (PKP)                              | 8.57             |
transcriptome derived by RNAseq. These included KRT16, MMP1, Plakophilin (PKP), and Cellular Retinoic Acid Binding Protein 2 (CRABP2), the latter of which was proposed as a putative biomarker for lung adenocarcinoma. There was also considerable overlap with the down-regulated genes (data not shown). A recent RNAseq whole transcriptome study of six normal, adenocarcinoma in situ and invasive adenocarcinoma samples also identified CRABP2 as up-regulated in adenocarcinoma in situ compared to normal lung, strongly implicating it in NSCLC progression.

**Pathways differentially expressed in Stage I and Stage II/III NSCLC adenocarcinomas**

To correlate differential gene expression with the biological pathways that are affected, gene enrichment analysis was performed. For Stage I tumours (Table 3) two major up-regulated KEGG pathways were identified: 34 genes in the cancer pathway and 13 in the more restricted small cell lung cancer pathway.

For Stage II/III tumours, two additional more specific cancer pathways were implicated: ECM-receptor interaction and focal adhesion, indicating the increased emphasis on cell migration and invasion in the later stage tumours. These pathways included many structural proteins (collagens, integrins, laminins, actinin4 ACTN4, tenascin C) as well as signalling molecules such as ERBB2, thrombopsondins 2 and 3, WNT5B, KRAS, BIRC2, cyclin D1, MAP kinase 9, protein phosphatase 1 PP1CA) and beta catenin (Supplemental Table 3). The effect on WNT, KRAS, PI3K-AKT, and MAPK signalling pathways would result in increased cell proliferation whereas increased ERBB2 signalling and anti-apoptotic protein BIRC2 would result in increased cell survival see Figure 1.

<table>
<thead>
<tr>
<th>Pathways in cancer</th>
<th>34</th>
<th>2.11E-06</th>
<th>3.29e-05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small cell lung cancer</td>
<td>13</td>
<td>1.91E-04</td>
<td>9.0e-04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPAR signaling pathway</th>
<th>15</th>
<th>1.98E-06</th>
<th>1.49E-05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkinson’s disease</td>
<td>18</td>
<td>6.24E-05</td>
<td>5.15E-05</td>
</tr>
<tr>
<td>Ascorbate and aldarate metabolism</td>
<td>7</td>
<td>7.02E-05</td>
<td>2.0E-04</td>
</tr>
<tr>
<td>Drug metabolism</td>
<td>12</td>
<td>9.44E-05</td>
<td>4.0E-04</td>
</tr>
<tr>
<td>Pentose and glucuronate interconversions</td>
<td>7</td>
<td>1.01E-04</td>
<td>2.0E-04</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>17</td>
<td>2.55E-04</td>
<td>2.0E-04</td>
</tr>
<tr>
<td>Glycolysis / Gluconeogenesis</td>
<td>11</td>
<td>3.36E-04</td>
<td>1.0E-03</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>20</td>
<td>5.11E-04</td>
<td>7.0E-04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pathways in cancer</th>
<th>43</th>
<th>1.07E-06</th>
<th>4.56e-06</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM-receptor interaction</td>
<td>18</td>
<td>6.10E-06</td>
<td>5.66e-06</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>17</td>
<td>2.57E-05</td>
<td>3.23e-05</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>28</td>
<td>4.47E-05</td>
<td>3.23e-05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPAR signaling pathway</th>
<th>14</th>
<th>1.67E-04</th>
<th>6.5e-03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate and aldarate metabolism</td>
<td>7</td>
<td>2.96E-04</td>
<td>6.5e-03</td>
</tr>
<tr>
<td>Drug metabolism</td>
<td>12</td>
<td>6.99E-04</td>
<td>1.66e-02</td>
</tr>
<tr>
<td>Arginine and proline metabolism</td>
<td>11</td>
<td>9.33E-04</td>
<td>1.66e-02</td>
</tr>
</tbody>
</table>

Table 3: Enrichment analysis using Web Gestalt (WG) and DAVID of differentially regulated genes in Stage I and Stage II/III NSCLC adenocarcinomas relative to control RNA. #, number of genes; p, p value.
In addition, up-regulation of ACTN4, PP1CA and DIA1 gene expression would positively impact regulation of the actin cytoskeleton and cell motility.

Both Stage I and Stage II/III tumours showed enrichment in down-regulated genes involved in PPAR signalling and various metabolic pathways (Table 3). PPAR γ has been implicated as a tumour suppressor in NSCLC, and xenograft models of lung cancer show that it inhibits lung tumour cell proliferation and growth through a variety of metabolic activities.42 Down-regulation of the anti-tumour PPAR signalling may enhance the ability of NSCLC tumours to grow and metastasize. PPAR ligands are under development as potential therapeutic agents for lung cancer.42

**Genes differentially expressed between Stage I and Stage II/III NSCLC adenocarcinomas**

Between Stage I and II/III tumours, there were 48 significantly differentially regulated genes (26 up-regulated and 22 down-regulated; p<0.01) (Figure 2; Supplemental Table 4).
PASD1 exhibited the greatest up-regulation between groups (9.4-fold) whereas the remainder were between 1.4 and 2.9-fold up-regulated. PASD1 is an immunogenic "cancer testis antigen" that is thought to function as a transcription factor and is associated with cancer of the small intestine, colon, lung, head and neck as well as hematopoietic malignancies. It shows promise as a target for immunotherapy against various hematopoietic cancers. PASD1 levels were significantly higher in H1299, a commonly used NSCLC cell line, than other cell lines and our results confirm that targeting it could also be a promising therapeutic approach in NSCLC. On the other hand, LCOR showed the greatest down-regulation (5.9-fold) between groups with the remaining 21 genes showing between 1.6 and 4-fold down-regulation. LCOR is a ligand-dependent co-repressor of various nuclear hormone receptors and has recently been shown to regulate transcription of CDKN1A, which encodes the cell cycle regulator p21, and the cell adhesion molecule, E-cadherin; reduced expression would promote transcription of cancer-related genes. Although this effect of LCOR on CDKN1A and E-cadherin has only been reported in cell lines and not yet for lung cancer, our studies suggest that this regulatory network is important in progression of NSCLC.

At a less stringent cut-off (p<0.05), there were 373 significantly differentially regulated genes between Stage I and II/III tumours (178 up-regulated and 195 down-regulated; Supplemental Table 5) of which the top 25 are shown in Table 4.

<table>
<thead>
<tr>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Ratio</td>
</tr>
<tr>
<td>Surfactant, pulmonary-associated protein A2 (SFTPA2)</td>
<td>9.94</td>
</tr>
<tr>
<td>PAS domain containing 1 (PASD1)</td>
<td>9.41</td>
</tr>
<tr>
<td>Nuclear receptor co-activator 7 (NCOA7)</td>
<td>4.93</td>
</tr>
<tr>
<td>Surfactant, pulmonary-associated protein D (SFTPD)</td>
<td>3.92</td>
</tr>
<tr>
<td>Matrix GlA protein (MGF)</td>
<td>3.37</td>
</tr>
<tr>
<td>Member RAS oncogene family (RAB2A)</td>
<td>3.33</td>
</tr>
<tr>
<td>PHD finger protein 11 (PHF11), variant 2</td>
<td>3.19</td>
</tr>
<tr>
<td>Src-like-adaptor (SLA), variant 3</td>
<td>3.06</td>
</tr>
<tr>
<td>Synovial sarcoma, X breakpoint 1 (SSX1)</td>
<td>3.05</td>
</tr>
<tr>
<td>Tubulin, alpha 1a (TUBA1A)</td>
<td>3.04</td>
</tr>
<tr>
<td>Ras-related associated with diabetes (RRAD)</td>
<td>3.03</td>
</tr>
<tr>
<td>Keratin associated protein 19-2 (KRTAP19-2)</td>
<td>2.98</td>
</tr>
<tr>
<td>Heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1), variant 2</td>
<td>2.96</td>
</tr>
<tr>
<td>Archaemetazincins-2 (AMZ2), variant 1</td>
<td>2.94</td>
</tr>
<tr>
<td>Ribosomal protein S4, X-linked (RPS4X)</td>
<td>2.85</td>
</tr>
<tr>
<td>Pinin, desmosome associated protein (PNN)</td>
<td>2.81</td>
</tr>
<tr>
<td>Leptin receptor overlapping transcript (LEPROT)</td>
<td>2.81</td>
</tr>
<tr>
<td>Leukotriene A4 hydrolase (LTA4H)</td>
<td>2.77</td>
</tr>
<tr>
<td>Calpain 2, (mII) large subunit (CAPN2)</td>
<td>2.74</td>
</tr>
<tr>
<td>Peptidylglycine alpha-amidating monoxygenase (PAM), variant 1</td>
<td>2.73</td>
</tr>
<tr>
<td>Niemann-Pick disease, type C2 (NPC2)</td>
<td>2.69</td>
</tr>
<tr>
<td>Dynet, light chain, LC8-type 1 (DYNLL1), variant 3</td>
<td>2.68</td>
</tr>
<tr>
<td>Histone cluster 1, H2bK (HIST1H2BK)</td>
<td>2.66</td>
</tr>
<tr>
<td>LSM1 homolog, U6 small nuclear RNA associated (LSM1)</td>
<td>2.66</td>
</tr>
<tr>
<td>Inositol 1,4,5-trisphosphate 3-kinase C (ITPKC)</td>
<td>2.65</td>
</tr>
</tbody>
</table>

Table 4: Top 25 differentially regulated genes in Stage II/III compared to Stage I NSCLC adenocarcinomas (identified using MeV at p<0.05).
Published gene expression studies have indicated that aggressive lung adenocarcinomas have higher levels of cell proliferation-related genes including cyclins, transcription factor TTF-1, surfactant SFTP B and those involved in immunological function. As mentioned above, TTF-1 regulates the transcription of CEACAM6 and surfactant-associated proteins. Both SFTP A2 and SFTP D were in the top five genes up-regulated in Stage II/III compared to Stage I tumours (Table 4) and cyclin I was up-regulated 2.56-fold in Stage II/III compared to Stage I tumours. MGP is a mesenchymal gene encoding an extracellular matrix protein and RT-PCR, ELISA and immunohistochemistry have shown that is over-expressed in recurrent gliomas and associated with poor outcome. It has not thus far been studied in lung cancer, and may provide a novel biomarker for lung recurrence. Immunohistochemistry, qRT-PCR, Western blot analysis and RNA interference have shown that LSM1 functions as an oncogene in the progression of lung cancer and members of the Ras and Src oncogene families (RAB2A, RRAD and SLA) were also up-regulated. Breast tumours shown to over-express RRAD by immunohistochemistry and Western blotting are of higher grade, size and nodal involvement and have poor prognosis. SLA mediates cell migration and invasion through integrin signalling by Src and FAK tyrosine kinases and in vitro over-expression studies have shown that it is also a negative regulator of T and B cell-mediated responses, which are crucial for anti-tumour immunity. Microarray, reverse phase protein array, ELISA and immunohistochemistry analyses have shown that patients with lung adenocarcinomas that express genes associated with an active immune response, such as RANTES, MIP-1 beta and STAT5 have better outcomes. Consistent with this is the 2.7-fold lower STAT5B expression in Stage II/III than Stage I tumours in our study.

Pathways differentially expressed between Stage I and Stage II/III NSCLC adenocarcinomas

Later-stage tumours showed enrichment in pathways mostly involved in transcription, translation, mitochondrial electron transport chain, and actin cytoskeleton organization (Supplemental Table 6A). This reflects the overall higher metabolism and growth rate of more advanced cancers. Interestingly, there was also enrichment in genes in the IL-6 signaling pathway; these included phospholipase C gamma subunit (PLCG1), FYN oncogene, ras-related small GTP-binding protein (RAC1), heat shock protein 90kDa alpha A1 (HSP90AA1), and protein phosphatase2 regulatory subunit B, gamma (PPP2R2C). FYN is a tyrosine kinase that has been implicated in the control of cell growth. HSP90AA1 is a chaperone for tyrosine kinases EGFR, MET and ALK, all of which are oncogenic drivers of lung cancer. PLCG1 is responsible for intracellular transduction of receptor-mediated tyrosine kinase activators. RAC1 is involved in control of cell growth/cytoskeletal reorganization, and PPP2R2C is also involved in tumour signal transduction pathways. IL-6 is present in the tumour microenvironment and induces tumour proliferation, angiogenesis and resistance to chemotherapy; it has recently been shown by tissue microarray analysis to be a novel prognostic biomarker in NSCLC. CD88, which is a high-affinity receptor for C5a that is widely expressed on lung epithelial cells, is up-regulated in Stage II/III (3.6-fold) compared to Stage I (2.3-fold) tumours. Over-expression of this receptor, assessed by tissue microarray analysis, correlates with down-regulation of E-cadherin expression and lymph node metastasis in NSCLC patients. Stage II/III vs. Stage I tumours did not show statistically significant enrichment in any pathways and there was only borderline enrichment in some GO biological processes (Supplemental Table 6B).

CONCLUSIONS

By comparing the transcriptomes of early and later-stage NSCLC adenocarcinomas, we have uncovered information on pathways that are involved in recurrence in early-stage lung cancer. Many of the hallmarks of cancer such as proliferative signalling, angiogenesis, invasion and metastasis were evident in the sets of genes we identified. A significant number of the differentially regulated genes participated in closely related processes, helping to validate our results. Transcript data reflected the overall higher growth rate of more advanced cancers and the involvement of pathways involved in actin cytoskeleton modelling and cell migration. While the small number of samples analysed in this study makes it difficult to make strong conclusions, we have nonetheless found concordant results with other reports that used a variety of complementary molecular techniques and uncovered possible pathways underlying recurrence. This study provides interesting novel leads to be followed up on in larger prospective investigations.

ACKNOWLEDGEMENT

We gratefully acknowledge the technical assistance of Jeffrey Gallant, Evelyn Teh and Jason Williams and bioinformatics analysis by Susanne Penny, National Research Council (NRC). We thank John Fris, database manager of the Lung Tumor Bank at Capital District Health Authority, for collating clinical annotations. Funding was provided by NRC and Dalhousie University Departments of Pathology and Surgery.

REFERENCES


The Impact of VAP Staff Education on VAP Morbidity and Mortality in Alexandria University

Elmenshawy AM1, Elbadawy TH2, Abu khaber H3, Hafez SF4, Fayed AM1 and Ibrahim EH5*

1Department of Critical Care Medicine, Alexandria University, Egypt
2Department of Cardiology and Angiology, Alexandria University, Egypt
3Department of Anesthesia and Surgical Intensive Care, Alexandria University, Egypt
4Department of Medical Microbiology and Immunology, Alexandria University, Egypt
5Department of Chest Diseases, Alexandria University, Egypt

ABSTRACT

Background: Staff education had several success stories in reducing Ventilator-associated Pneumonia (VAP) rate. However, the stability of supplies and the top management support were not addressed in most of these studies. In addition, both were considered essential in several reviews.

Aim: To determine the efficiency (VAP rate) and efficacy (mechanical ventilation morbidity and mortality) of VAP staff education with deficient supplies and lack of top management support.

Methods: Quasi-experimental study with before and after prospective cohort in two medical/surgical ICUs of Alexandria university affiliated hospitals during the period from September 2007 till May 2013. The intervention phase included the provision of supplementary supplies, interactive education for physicians and nurses followed by a VAP campaign. All VAP episodes not only the first one was included.

Results: A total of 598 patients were enrolled in the study. The adherence to expanded VAP bundle significantly increased in the post-intervention phase as follows; head of bed elevation (from mean of 40 to 100% with p=0.001), oral care (from mean of 20 to 100% with p=0.001), daily sedation vacation (from mean of 56.5 to 91% with p=0.001), daily assessment of weaning (from mean of 9 to 25% with p=0.03), peptic ulcer prophylaxis (from mean of 83 to 100% with p=0.001), DVT prophylaxis (from mean of 82 to 100% and p=0.001), cuff pressure measurement (from mean of 9 to 60% with p=0.001), and hand hygiene (from mean of 8 to 28.5% with p=0.001). The VAP rate decreased significantly by 35% (from 66.5 to 43 per 1000 MV days) with p= 0.002 and CI 9.73-37.15 in spite of significant increase of the ventilator utilization ratio (p <0.001) in the post-intervention phase. The MV, antibiotic and ICU days did not change significantly in the post-intervention phase. The distribution of organisms did not differ significantly between both groups (p=0.465). The sensitivity of most of carbapenems and β-lactam/β-lactamase inhibitors to Acinetobacter, Klebsiella and Pseudomonas decreased significantly in the post intervention phase whereas the sensitivity of vancomycin to Staphylococcus aureus remained the same.

Conclusions: In spite of the lack of top management support and fluctuating supplies, VAP staff education was still efficient in reducing VAP without affecting mortality or MV days or ICU length of stay.

ABBREVIATIONS: VAP: Ventilator-associated Pneumonia; MDRP: Multidrug Resistant Pathogens.
INTRODUCTION

Ventilator-associated Pneumonia (VAP) is peculiar from other Hospital Acquired Infections (HAIs) in several aspects. VAP has the highest prevalence, complex pathogenesis (and therefore multiple preventive procedures) and controversies in diagnosis and treatment which hindered accurate diagnosis and optimal therapy. In addition, the high prevalence of Multidrug Resistant Pathogens (MDRP) further complicates the VAP management and magnifies the morbidity and mortality impact of VAP. Therefore prevention is the most effective treatment.

Several evidence based interventions proved to reduce VAP which have been incorporated into guidelines by several organizations. Implementing evidence-based guidelines into consistent delivered care at bedside still remains a challenge. Staff education programs for single procedures or selected bundle of several interventions significantly reduced VAP rate in several studies. Furthermore, staff education and involvement was recommended in several guidelines.

The top management support and availability of supplies were considered crucial for a successful VAP program. After the initial failure of VAP reduction in two ICUs of Alexandria Main university hospital which was attributed to deficient supplies, lack of top management support and passive education. In addition to repeated failure to obtain funding or top management support for the VAP program, we conducted a study with the purpose to assess the efficiency (VAP rate) and efficacy (Mechanical ventilation morbidity and mortality) of VAP staff education without top management support and full availability of supplies.

METHODS

Prior to the initiation of the study, the approval of the Alexandria University Ethical Committee on the general plan of the study, and the detailed patient management plan was taken. An informed consent was obtained before enrolment in the study (before completing the 48 hour period of mechanical ventilation).

Study location: This study was carried out in ICU1 and ICU3 of Critical Care Medicine department in Alexandria University affiliated Hospital, which is a major adult urban teaching, primary and tertiary care facility with 1900 beds, serving 3 governorates. Both ICU1 and ICU3 are 15 beds; medical/surgical with approximately 1269 and 610 admissions per year respectively.

Through FOCUS PDSA cycle (which is considered the primary engine of this study), we first found an opportunity in the failure of previous study to reduce VAP rate. Second, we clarified the current process through reviewing outcomes and limitation of the previous study. Fourth, we understood the causes of process variation through reviewing local VAP studies with bacterial investigation of VAP pathogenesis, and the proposed solutions in sampling, suctioning, oral hygiene, draining circuit condensate, hand hygiene and patient transport according to available resources. Fifth, we Selected the process improve ment using quasi-experimental study using before and after prospective cohorts with process and outcome indicators and cost-effectiveness data to evaluate the efficiency and efficacy of the VAP staff education and to be re-modelled with Plan Do Study Act cycle (Study design)

This study was performed after initial failure of passive education from September 2007 till June 2009 (involving four lectures 20 minutes each in physician lounge room, scientific meetings and limited bedside teaching for the residents) without availability of enough supplies to reduce VAP rate. The current intervention program was designed to overcome the limitations of the previous study followed by post-intervention phase which was compared to the pre-intervention phase of the previous study with collection of more patient data retrospectively for VAP risk stratification.


The intervention phase was optimized by avoiding certain limitations from the previous study providing supplementary supplies donated to the hospital to decrease the gap between the required and the available (such as alcohol and suction catheters, beds for semi-seating position, and cuff pressure manometers), providing lectures for nurses, together with bedside teaching, implementing regular endotracheal cuff pressure adjustment and blind mini BAL (bronchoalveolar lavage), utilizing interactive lectures to physicians, carrying out a hand hygiene then a VAP campaign which included two days workshop, skill stations, group discussions, VAP folder for self education, and a concentrated bedside teaching.

Study population: This study was conducted on all patients requiring continuous Mechanical Ventilation (MV) for more than 48 hours who were admitted to both units during the study period. Patients with VAP which was not acquired in our units or those whom were transferred to other units before weaning from the ventilator were excluded. The screening for potential participants were performed at least every 48 hours as well follows up of participants for the development of VAP and final outcome.

All patients in the pre and post-intervention phases were evaluated for baseline and VAP risk stratification data (age, gender, primary diagnosis, severity of illness by Acute Physi-
ological and Chronic Health Evaluation II “APACHE II”, history of chronic respiratory illness and Glasgow Coma Scale “GCS”). They were followed up for the development of VAP by modified American College of Chest Physicians (ACCP) criteria for diagnosis of VAP: “New and persistent (≤72 h) radiological infiltrate with two or more of the following: Fever (increase in core temperature of > 1°C and a core temperature >38.3°C or <35.5°C), leucocytosis (25% increase in circulating leucocytes from baseline and a value >10,000 cells/ml), purulent tracheal aspirate or sputum” until death, discharge out of ICU or transfer to another ICU.

VAP rate per 1000 ventilator days (primary outcome), adherence to VAP preventive practices (expressed as percentage of positive observation during the scheduled ICU visits), culture results of VAP samples obtained by Bronchoscopic Broncho-Alveolar Lavage (BAL), or modified blind mini-BAL (by using saline filled catheter instead of protected catheter and guiding it into desired bronchus through neck positioning then performing a lavage), MV days and ICU Length of Stay (LoS), and outcome (secondary outcomes) were collected in the pre and post-intervention phases. VAP rate was tracked through control charts (U chart).

STATISTICAL ANALYSIS OF THE DATA

Data was fed to the computer and analyzed using IBM SPSS software package version 20. Qualitative data was described using number and percent. Quantitative data was described using mean and standard deviation for normally distributed data while abnormally distributed data was expressed using median, minimum and maximum. Comparison between different groups regarding categorical variables was tested using Chi-square test. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D’Agstino test, also Histogram and QQ plot were used for vision test. If it revealed normal data distribution, parametric tests were applied. If the data was abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent populations was done using independent t-test. For abnormally distributed data, comparison between two independent populations was done using Mann Whitney test. Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

A total of 2560 patients were screened in both ICU1 and ICU3 (Figure 1). After exclusion of non-MV patients, MV for ≥48 hours, and patients with VAP acquired in other units, 598 patients were enrolled in the pre and post-intervention phases. One patient in the ICU3 stayed on MV for 276 days till the end of the study who was excluded as an extreme value. The rates of loss to follow up were 4.4 and 4.3% in the pre and post-intervention phases respectively. The number and qualification of physicians caring for patients (5-6/unit/shift) did not differ between both groups. Similarly, the number of non-registered nurses in the morning and evening shifts did not differ between both groups (4-5/unit in the morning and 1-2 in the evening and night shift). Whereas, the number of registered nurses dropped to the half in the post-intervention phase in the evening and night shift (0-1). The residents who were involved in the formal teaching (lectures or workshop) represented 80-87% of all residents, whereas the nursing staff who was involved in workshops represented 90-95% of all nursing staff (registered and non-registered nurses) in both units.

<table>
<thead>
<tr>
<th>Pre-intervention phase</th>
<th>Post-intervention phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1459 patients admitted in ICU1 and ICU3</td>
<td>1101 patients admitted in ICU1 and ICU3</td>
</tr>
<tr>
<td>560 (38%) MV patients screened</td>
<td>626 (57%) MV patients screened</td>
</tr>
<tr>
<td>285 (51% of MV) did not meet inclusion criteria</td>
<td>275 (44% of MV) did not meet inclusion criteria</td>
</tr>
<tr>
<td>275 patients (49% of MV) enrolled</td>
<td>351 (56%)of MV) patients enrolled</td>
</tr>
<tr>
<td>2 (0.7% of enrolled) transferred on MV</td>
<td>4 (1% of enrolled) transferred on MV</td>
</tr>
<tr>
<td>104 (4% % of enrolled) loss to follow up</td>
<td>11 (3%) of enrolled loss to follow up</td>
</tr>
<tr>
<td>263 patients finally enrolled</td>
<td>335 patients finally enrolled (1 excluded as extreme value on MV)</td>
</tr>
</tbody>
</table>

The demographic characteristics and VAP risk stratification data were similar between the pre and post-intervention groups except for the admission diagnosis which differed significantly in the post-intervention phase (decreased cardiac cases and increased surgical cases) (Table 1).

Primary outcome: Among the 263 patients constituting the study sample in the pre-intervention phase, 132 patients fulfilled the criteria of clinically defined VAP using ACCP clinical criteria with an incidence of 50.2%. Out of the 132 clinically defined VAP cases, there were 116 laboratory (lab) confirmed VAP (4 were not sampled, 4 sterile samples, and 8 contaminated). The incidence of lab confirmed VAP was 44.1% (Table 1). The sensitivity of the lab to verify clinical diagnosis was 87.9%.

APACHE II: Acute Physiological and Chronic Health Evaluation II GCS: Glasgow Coma Scale Lab: Laboratory#: Chi square test $: Mann Whitney test &: Student t-test*: Statistically significant at $p \leq 0.05$

Among the 335 patients constituting the study sample in the post intervention phase, 138 patients fulfilled the criteria of clinically defined VAP using ACCP clinical criteria with an incidence of 41.2%. Out of the 138 clinically defined VAP, 116 were lab confirmed VAP (4 were not sampled, 9 sterile
<table>
<thead>
<tr>
<th></th>
<th>Pre-intervention (n= 263)</th>
<th>Post-intervention (n= 335)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>122 (46.4%)</td>
<td>181 (54.0%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Female</td>
<td>48.0 (1.0 – 66.0)</td>
<td>52.0 (1.0 – 91.0)</td>
<td>0.099</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnosis type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trauma</td>
<td>44 (16.7%)</td>
<td>53 (18.5%)</td>
<td>0.961</td>
</tr>
<tr>
<td>Cardiac</td>
<td>57 (21.7%)</td>
<td>20 (6.0%)</td>
<td>0.179</td>
</tr>
<tr>
<td>Respiratory</td>
<td>7 (2.7%)</td>
<td>58 (17.3%)</td>
<td>&lt;0.005*</td>
</tr>
<tr>
<td>Surgical</td>
<td>31 (11.8%)</td>
<td>27 (8.1%)</td>
<td>0.055</td>
</tr>
<tr>
<td>Neurological</td>
<td>0 (19.0%)</td>
<td>52 (15.5%)</td>
<td>0.190</td>
</tr>
<tr>
<td>Medical</td>
<td>32 (12.2%)</td>
<td>91 (27.2%)</td>
<td></td>
</tr>
<tr>
<td>Toxicological</td>
<td>19.0 (4.0 – 42.0)</td>
<td>34 (10.1%)</td>
<td></td>
</tr>
<tr>
<td>APACHE score</td>
<td>90.24 ± 2.94</td>
<td>63 (18.8%)</td>
<td>0.949</td>
</tr>
<tr>
<td>History of chronic respiratory illness</td>
<td>9.24 ± 2.94</td>
<td>63 (18.8%)</td>
<td>0.949</td>
</tr>
<tr>
<td>GCS</td>
<td>132 (50.2%)</td>
<td>9.41 ± 2.40</td>
<td>0.467</td>
</tr>
<tr>
<td>Clinically defined VAP</td>
<td>34 (25.8%)</td>
<td>138 (41.2%)</td>
<td>&lt;0.028*</td>
</tr>
<tr>
<td>Early onset</td>
<td>98 (74.2)</td>
<td>21 (15.2%)</td>
<td>0.005*</td>
</tr>
<tr>
<td>Late onset</td>
<td>116 (44.1%)</td>
<td>117 (84.8)</td>
<td>0.554</td>
</tr>
<tr>
<td>Lab confirmed VAP</td>
<td></td>
<td>116 (34.6%)</td>
<td>0.018*</td>
</tr>
<tr>
<td>Number of VAP attack of MV pts</td>
<td>100 (75.8%)</td>
<td>107 (77.5%)</td>
<td>0.121</td>
</tr>
<tr>
<td>Single (1 attack)</td>
<td>32 (24.2%)</td>
<td>107 (77.5%)</td>
<td></td>
</tr>
<tr>
<td>Multiple (≥2 attacks)</td>
<td>6.0 (2.0 – 67.0)</td>
<td>31 (22.5%)</td>
<td>0.249</td>
</tr>
<tr>
<td>MV days</td>
<td>9.0 (2.0 – 110.0)</td>
<td>6.0 (2.0 – 76.0)</td>
<td>0.119</td>
</tr>
<tr>
<td>ICU stay</td>
<td>8.0 (0.0 – 96.0)</td>
<td>9.0 (2.0 – 147.0)</td>
<td>0.185</td>
</tr>
<tr>
<td>AB days</td>
<td>8.0 (0.0 – 99.0)</td>
<td>8.0 (0.0 – 99.0)</td>
<td>0.089</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>103 (39.2%)</td>
<td>183 (54.6%)</td>
<td>0.127</td>
</tr>
<tr>
<td><strong>Distribution of organisms</strong></td>
<td>47 (22.6%)</td>
<td>40 (15%)</td>
<td>0.465</td>
</tr>
<tr>
<td>Gram positive</td>
<td>27 (13%)</td>
<td>54 (19.9%)</td>
<td></td>
</tr>
<tr>
<td>staphylococcus aureus</td>
<td>161 (77.4%)</td>
<td>40 (15%)</td>
<td>0.465</td>
</tr>
<tr>
<td>Gram negative</td>
<td>54 (26%)</td>
<td>218 (80.1%)</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>49 (23.6%)</td>
<td>47 (17%)</td>
<td></td>
</tr>
<tr>
<td>Pseudomonasaeroginosa</td>
<td>11 (2.9%)</td>
<td>55 (20%)</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>74 (27%)</td>
<td>74 (27%)</td>
<td></td>
</tr>
<tr>
<td>Mono versus poly-microbial</td>
<td>97 (73.5%)</td>
<td>88 (58.3%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>VAP</td>
<td>35 (26.5%)</td>
<td>(n= 151)</td>
<td></td>
</tr>
<tr>
<td>Mono-microbial</td>
<td>63 (41.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly-microbial</td>
<td>88 (58.3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Comparison between pre and post-intervention groups according to demographic characteristics, VAP risk stratification, primary and secondary outcomes.
samples, 9 contaminated) with an incidence of 34.6% and a sensitivity of lab verification for clinical diagnosis was 84.1% (Table 1). Therefore, there was statistically significant decrease in the incidence of clinically defined VAP by 17.9% (p = 0.028) and lab confirmed VAP by 21.5% (p = 0.018) in the post-intervention phase (Table 1). The incidence of early onset VAP decreased significantly (from 25.8 to 15.2% with p = 0.005), on the other hand the incidence of late onset, single and multiple VAP decreased insignificantly (p = 0.554, 0.121, and 0.249 respectively) in the post-intervention study group (Table 1).

During the 9 month of the pre-intervention phase, a total of 179 VAP episodes occurred during a total of 2740 ventilator days, with a VAP rate of 66.5 per 1000 ventilator days. Following the intervention, a total of 200 VAP episodes occurred during a total of 4619 ventilator days, and VAP rate dropped significantly to 43 with a decrease of 35.3% and p value of 0.002(mean difference of 23.4 and confidence interval of 9.7-37.1),although the DU ratio increased significantly by 51.3% and p =0.001 (Figure 2 and Table 2).

**Figure 2:** Control chart of VAP Rate per 1000 ventilation days in the pre and post-intervention phases in ICU1 and 3

<table>
<thead>
<tr>
<th></th>
<th>Pre-intervention (n= 9 months)</th>
<th>Post-intervention (n= 9 months)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAP rate per 1000 ventilator days</td>
<td>66.49 ± 16.75</td>
<td>43.05 ± 9.79</td>
<td>0.002*</td>
</tr>
<tr>
<td>Ventilation utilization ratio</td>
<td>0.39 ± 0.04</td>
<td>0.59 ± 0.03</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Data was expressed as Mean ± SD. for normally distributed data

**Table 2:** Comparison between the pre and post-intervention groups according to VAP rate and ventilation utilization ratio

**Short term outcome:** There was a highly significant increase in the compliance or adherence to VAP preventive practices in the post-intervention study group as follows: head of bed elevation (from mean of 40 to 100%, p < 0.001), oral care (from mean of 20 to 100%, p < 0.001), daily sedation vacation (from mean of 56.5 to 91%, p <0.001), daily assessment of weaning (from mean of 9 to 25%, p =0.03), peptic ulcer prophylaxis (from mean of 83 to 100%, p < 0.001), DVT prophylaxis (from mean of 82 to 100%, p < 0.001), cuff pressure measurement (from mean of 9 to 60%, p <0.001), and hand hygiene (from mean of 8 to 28.5%, p =0.001) (Figure 3).

**Secondary Outcome:** There was insignificant difference between the pre and post-intervention groups as regards duration of mechanical ventilation, ICU length of stay, antibiotic days, and mortality with p=0.119, 0.185, 0.089 and 0.127 respectively (Table 1).

**MICROBIOLOGY AND SENSITIVITY PATTERN**

The distribution of organisms did not differ significantly between the pre and post-intervention groups with p value of 0.465, on the other hand the incidence of poly-microbial VAP increased significantly in the post-intervention phase with p value <0.001 (Table 1). The most common organisms in the pre-intervention phase were Klebsiella, Pseudomonas followed by Staphylococcus aureus, while in the post-intervention phase were Acinetobacter, Pseudomonas, Klebsiella followed by Staphylococcus aureus (Table 1).

The antibiotic sensitivity pattern of Klebsiella decreased significantly to imipenem, piperacillin-tazobactam, cefoperazone-sulbactam, and amikacin the post-intervention phase. Similarly, the sensitivity of Pseudomonas decreased significantly to carbapenems, β-lactam β-lactamase inhibitor combinations, third and fourth generation cephalosporins and aminoglycosides in the post-intervention phase. The Acinetobacter sensitivity decreased significantly to carbapenems, piperacillin-tazobactam and trimethoprim sulfamethoxazole in the post-intervention phase. On the other hand the Staphylococcus aureus sensitivity to vancomycin still remained the same (100%).

**Figure 3:** Comparison between the pre and post intervention phases according to compliance to VAP preventive practices (expressed as percentage of positive observation) in both ICUs.
DISCUSSION

Several effective interventions are not always adopted despite evidence supporting their use,50-60 and this is not different when it comes to VAP prevention.61-64 Although guidelines have been used to promote consistency and reduce variation in clinical practice, the successful implementation of any guideline is by no means assured and is dependent upon many factors, including implementation strategies that need to be tailored to the local situation.8 Educational program has been utilized in VAP prevention in different aspects, mainly education for single procedures as hand hygiene,9 oral hygiene,10 sedation protocol11 or staff education for either Institute for Healthcare Improvement (IHI) bundle45 or its modification46-47 or other selected ventilator bundle which includes several interventions for VAP prevention.48-50

The baseline characteristics and VAP risk stratification data (age, sex, APACHE II score, GCS, and history of chronic respiratory illness) were homogeneous between the pre and the post-intervention groups except for the admission diagnosis which differed significantly with a marked decrease of cardiac cases and increase of post-surgical cases in the post-intervention group. This was directly attributed to the diversion of most of acute coronary syndrome cases to new cardiac ICU during the intervention phase. The long lag between the pre and the post-intervention phases in the present study during which the circumstances related to admission in both units had changed, might contributed to these discrepancies in diagnosis type. However, neither the diagnosis type nor the place from which the patient was transferred (medical ward, scheduled surgical or emergency surgery) did increase risk of VAP in three studies.51,52,53

The DU ratio (a measure of invasive practices in a unit which constitutes an extrinsic risk factor for VAP54 and a marker for severity of illness of patients55 was highly significantly increased in the post-intervention group by 51%. This might be explained by the increased number of ventilators in the post-intervention phase. In addition, the registered nurse/patient ratio dropped by 50% in the evening and night shifts in the post-intervention phase which might have reduced patient care. This might have contributed to the increased risk of VAP in the post-intervention group as a result of shortage of competent nursing staff55-56 and the highly significantly increased DU ratio. In spite of this, the VAP rate was decreased significantly by 35% as well as the clinically and laboratory confirmed VAP and the early onset VAP. On the other hand, the incidence of late onset, single and multiple VAP showed an insignificantly decrease.

Several educational program have reduced VAP rate such as Zack et al by 57%, Babcock et al by 38-61%, Apisarnthanarak et al by 59%, Salahuddin et al by 51%, Leblebicioglu et al by 46%, Bouadma et al by 43%, Blamoun et al by 100%, Berriel-Cass et al by 60% Lansford et al by 59.4%, and International Nosocomial Infection Control Consortium (INICC) multimodal program by 30.7-79% in several studies.21,23,62-64 The discrepancy between the adherence rate to VAP preventive practices and VAP rate reduction in those studies precluded any relation between the adherence to our VAP bundle and VAP rate reduction in this study. Similarly Zilberberg et al65 revealed that the major methodological flaws in the design, reporting, and results of the published studies precluded any conclusive statements about bundle compliance and VAP reduction. Similarly, Beattie et al66 failed to preclude any link between bundle compliance and development of VAP which they attributed to retrospective nature and small sample size of their study.

VAP bundle adherence, especially the overall adherence, is not the only determinants of the magnitude of VAP reductions. Other factors are important like working policy and procedures, nursing staffing, and equipment supply65 which might explain different effect in these studies. Another explanation is that these VAP prevention programs utilized different educational (and sometime quality tools) to implement different combination of VAP preventive practices, this might have explained the different effects between studies. This was also illustrated by Marra et al67 who tested the effects of different bundles introduced sequentially and had different results.

As in our earlier study that failed to reduce VAP rate, some educational studies also did not reduce VAP rate as Abbot et al14 and Bloos et al15 in spite of utilizing a variety of educational tools. Similarly Bingham et al,13 did not reduce VAP which they attributed to frequent changes in personnel and leadership in the organization. In addition Hawe et al,68 Papadimos et al,69 Bigham et al,70 and Coancour et al,71 after failure of passive education to improve bundle compliance and VAP rate, active education significantly improved bundle compliance and VAP rate. This suggests that addition of procedural or quality tool to interactive education significantly improved program efficiency.69

Morris et al72 reduced clinically defined and laboratory confirmed VAP, while Rosenthal et al,64 Bouadma et al8 and Bigham et al80 reported a significantly decreased clinically defined VAP only. Babcock et al78 and Apisarnthanarak et al16 did not affect time of onset of VAP. It might be suggested that the high incidence of early onset of VAP in the pre-intervention phase (known to be caused by intubation related aspiration) was more feasible to correction by educational program which was higher than those studies. The success stories of VAP reduction are often reported from hospitals with high baseline VAP.73 On the other hand, Omrane et al74 found that early onset VAP significantly decreased and late onset VAP significantly increased by educational program but through incidence density (per 1000 ventilation days). To the best of our knowledge, only Bouadma et al58 reported decreased incidence of single and multiple VAP
without statistical processing.

In this study, the secondary outcomes (ICU, MV and AB days and MV mortality) did not change significantly with staff education. Leblebicioglu et al,20 Rosenthal et al,62 Omrane et al74 Morris et al,72 and Bigham et al70 reported a significant decrease of hospital stay in the post-intervention phase. While, Abbot et al14 and Bloos et al15 reported a significant decrease of MV days despite unaffected ICU stay. Bouadma et al58 did not find difference in total MV days or hospital stay. Apisarnthanarak et al,16 Rosenthal et al,64 and Youngquist et al75 reported a significant decrease of hospital stay in the post-intervention phase. While, Abbot et al14 and Bloos et al15 reported a significant decrease of MV days despite unaffected ICU stay. Bouadma et al58 did not find difference in MV days inspite of significantly reduced ICU stay. Apisarnthanarak et al,16 Rosenthal et al,64 Bouadma et al58 Marra et al18 reported no significant difference of mortality rate with educational program. Several educational programs did not evaluate outcomes other than VAP rate.17-18,20 Hawe et al12 and Morris et al72 found a trend to reduced unit and MV mortality. Morris et al72 found that antibiotic days did not change in all MV patients with VAP education.

The educational program did not affect the microbiology of organisms causing VAP (gram positive or negative) in spite of increased incidence of poly-microbial VAP. Similarly, educational program by leblebicioglu et al,20 Rosenthal et al,64 and Babcock et al did not affect the microbiology of infections, suggesting that intervention improved ventilator management and care rather than eliminating a particular nosocomial reservoir of infection.18 While Apisarnthanarak et al,16 Morris et al,72 and Bouadma et al58 reported only an outbreak of acinetobacter, decreased rates of methicillin-resistant Staphylococcus aureus acquisitions, and higher trend of staphylococcus aureus in the post-intervention study group respectively. Although the antibiotic sensitivity of most common organisms decreased to most relevant antibiotics in this study, Leblebicioglu et al20 Rosenthal et al64 reported non-significant change of antibiotic resistance to Acinetobacter, Pseudomonas and Staphylococcus aureus between the pre and post-intervention groups. This might be explained by the high rates of antibiotic abuse in our units. It might also be suggested that the reducing effect of VAP educational program on MV days and ICU stay might have been reversed by the highly significant increase of MDRP. However we did not evaluate other factors affecting MV days, ICU stay as other hospital acquired infections, ICU performance, and iatrogenic ICU complications (Table 3).

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Klebsiella</th>
<th>P</th>
<th></th>
<th>Pseudomonas</th>
<th>p</th>
<th></th>
<th>Acinetobacter</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre (n= 54)</td>
<td>Post (n= 47)</td>
<td>0.273</td>
<td>Pre (n= 49)</td>
<td>Post (n= 55)</td>
<td>0.001*</td>
<td>Pre (n= 3)</td>
<td>Post (n= 74)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>45 (83.3%)</td>
<td>35 (74.5%)</td>
<td>0.273</td>
<td>36 (73.5%)</td>
<td>14 (25.5%)</td>
<td>&lt;0.001*</td>
<td>2 (66.7%)</td>
<td>5 (6.8%)</td>
</tr>
<tr>
<td>Impinen</td>
<td>47 (87.0%)</td>
<td>28 (59.6%)</td>
<td>0.002*</td>
<td>37 (75.5%)</td>
<td>10 (18.2%)</td>
<td>&lt;0.001*</td>
<td>3 (100.0%)</td>
<td>13 (17.6%)</td>
</tr>
<tr>
<td>Tazobactam-piperacillin</td>
<td>48 (88.9%)</td>
<td>18 (38.3%)</td>
<td>&lt;0.001*</td>
<td>39 (79.6%)</td>
<td>17 (30.9%)</td>
<td>&lt;0.001*</td>
<td>1 (33.3%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Ceftazidim</td>
<td>42 (77.8%)</td>
<td>7 (14.9%)</td>
<td>&lt;0.001*</td>
<td>33 (67.3%)</td>
<td>2 (3.6%)</td>
<td>&lt;0.001*</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>5 (9.3%)</td>
<td>2 (4.3%)</td>
<td>0.445</td>
<td>2 (4.1%)</td>
<td>0 (0.0%)</td>
<td>0.220</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>4 (7.4%)</td>
<td>2 (4.3%)</td>
<td>0.683</td>
<td>2 (4.1%)</td>
<td>0 (0.0%)</td>
<td>0.101</td>
<td>0 (0.0%)</td>
<td>1 (1.4%)</td>
</tr>
<tr>
<td>Cefepim</td>
<td>17 (31.5%)</td>
<td>10 (21.3)</td>
<td>0.248</td>
<td>16 (32.7%)</td>
<td>6 (10.9%)</td>
<td>0.007*</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Cefazidim</td>
<td>7 (13.0%)</td>
<td>6 (12.8%)</td>
<td>0.976</td>
<td>10 (20.4%)</td>
<td>4 (7.3%)</td>
<td>0.049*</td>
<td>0 (0.0%)</td>
<td>1 (1.4%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>18 (33.3%)</td>
<td>27 (57.4%)</td>
<td>0.015*</td>
<td>20 (40.8%)</td>
<td>8 (14.5%)</td>
<td>0.003*</td>
<td>1 (33.3%)</td>
<td>2 (7.7%)</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>15 (27.8%)</td>
<td>19 (40.4%)</td>
<td>0.180</td>
<td>18 (36.7%)</td>
<td>6 (10.9%)</td>
<td>0.002*</td>
<td>1 (33.3%)</td>
<td>8 (10.8%)</td>
</tr>
<tr>
<td>TMP-SMX</td>
<td>8 (14.8%)</td>
<td>9 (19.1%)</td>
<td>0.561</td>
<td>0 (0.0%)</td>
<td>1 (1.8%)</td>
<td>1.000</td>
<td>2 (66.7%)</td>
<td>3 (4.1%)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>21 (38.9%)</td>
<td>21 (44.7%)</td>
<td>0.556</td>
<td>20 (40.8%)</td>
<td>4 (7.3%)</td>
<td>&lt;0.001*</td>
<td>1 (33.3%)</td>
<td>5 (6.8%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>24 (45.9%)</td>
<td>13 (27.7%)</td>
<td>0.844</td>
<td>11 (22.4%)</td>
<td>4 (7.3%)</td>
<td>0.028*</td>
<td>0 (0.0%)</td>
<td>4 (5.4%)</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>3 (5.6%)</td>
<td>3 (6.4%)</td>
<td>1.000</td>
<td>4 (8.2%)</td>
<td>0 (0.0%)</td>
<td>0.049*</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Amoxicillin-clavuante</td>
<td>6 (11.1%)</td>
<td>8 (17.0%)</td>
<td>0.391</td>
<td>4 (8.2%)</td>
<td>0 (0.0%)</td>
<td>0.049*</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

Patients may have more than one organism in single VAP episode, so sum of organisms exceed the total number of VAP episodes

Table 3: Comparison of antibiotic sensitivity pattern of gram negative organisms implicated in VAP cases in the pre and post intervention groups in Critical Care Unit 1 and 3.

**Note:** TMP-SMX: trimetho primsulfamethoxazol
The costs of educational program were estimated not to exceed 1000 $ (conference rooms renting, folder and booklet materials printing), and additional infection control supplies not exceeding 3000$. There were no additional salaries for personnel sharing in the education program. Assuming a continued infection rate of 66.5 (of the pre-intervention phase) during the post-intervention phase, 302 VAP episodes would be expected to occur in the 4619 ventilator days in both units. Several studies measured the cost of a single VAP episode as low as 4,947-5,800 and up to 40,000$. As our educational program prevented 102 episodes in post-intervention study group. The cost saving of this educational program was estimated to be at least 0.5 million dollars. Using the same calculation; Babcock et al. prevented 98 episodes and consumed 592000$, and therefore had a cost-saving of 0.4 million dollars.

Similarly, Apisarnthanarak et al. reported a 37-45% reduction of mean hospital (4665 vs. 2935 with P =0.001) and monthly antibiotic costs (4769$ vs. 2622$ with p =0.001) with VAP prevention program. Zilberberg et al. concluded that the available data of VAP prevention program is still difficult for accurate cost-effectiveness studies because it relates to such important downstream outcomes of VAP prevention as the use of antibiotics and hospital length of stay.

Several reviews addressed the discrepancy between the results of VAP education as Jansson et al.22 who suggested that it might be due to the wide range of definitions of VAP applied, lack of a universal method of outcome evaluation, variations in the implementation strategies (i.e. in the execution and frequency of education), ventilator bundle and various complementary interventions (reminders, feedback etc.) Moreover, the effects of extraneous factors or other potential sources of bias on their findings were inadequately reported resulting in both practical and methodological difficulty.15

Klompas et al. attributed the paradox of VAP intervention (either a single procedure like oral hygiene, semi-sitting position, silver coated tubes or multiple procedures) to VAP misclassification; as it dramatically reduce VAP whereas most of them have no impact on clinical outcome (MV days, hospital stay, or mortality). As the sensitivity and specificity of microbiological diagnosis are only 50%-70% and 40%-95% respectively. This include non-infectious mimics of VAP with bacterial colonization which are easily affected by simple preventive measures.90

These reviews also concluded that active implementation strategies (i.e. educational programs) were linked to significant improvements in the overall adherence to ventilator bundles and a significant decrease in clinical outcomes: incidence of VAP, duration of mechanical ventilation and hospitalization costs. The opportunities for decreasing VAP rates seem to be greatest when multi-module programs were applied with an average reduction of more than 40%. Arabi et al. concluded that simple cost-effective measures reduced the incidence of VAP significantly in developing countries with limited healthcare resources. For this reasons, staff education and involvement was recommended in most VAP prevention guidelines as American Thoracic Society and Infectious Disease Society of America (I), Center of Disease and Control (IA), SHEA and Infectious Disease Society of America (A-II).

The pre-intervention VAP rate was higher than that reported in multicenter surveillance networks. The mean rates were 61 per 1000 ventilator-days in both ICUs. As compared to National Healthcare Safety Network 2008 (NHSN; formerly National Nosocomial Infections Surveillance [NNIS]) system, the 50th percentile of major teaching medical-surgical ICUs was 2.3, INICC 2008 mean VAP rate of medical/surgical ICU was 14.7, INICC 2008 mean VAP rate of medical/surgical ICU was 14.7, German Krankenhaus Infektions Surveillance System (KISS) surveillance system was 8.0 cases in all ICUs, and the French national surveillance system median rate in all ICUs was 16.4 cases per 1000 ventilator-days in 2008.

In the post-intervention phase, the mean VAP rates were 41 per 1000 ventilator days in both ICUs. As compared to NHSN 2011, the 50th percentile of major teaching medical-surgical ICU was 1, KISS mean VAP rate of 6.8. The large differences between our VAP rates and international VAP rate can be explained by several factors.

First: The NNIS and INICC utilized the CDC definitions while we utilized ACCP criteria for diagnosis. Skrupky et al. when compared the CDC and ACCP definition in the same study group showed large discrepancy (1.2 vs. 8.5 per 1000 ventilator days). Similarly the difference between the NNIS, KISS rates and the rates in France might be attributed to the different criteria for defining VAP across networks.

Second: Studies from limited-resource countries have shown that VAP rates were more than three-fold higher than those in developed countries, where lower-middle income countries had higher VAP rates than upper-middle-income countries (9.0 vs. 0.5 per 1000 MV-days). Thus this higher VAP rate might be related to limitations associated with socioeconomic factors: the ICU equipment is less advanced than in developed countries, medical resources are relatively insufficient, there are crowded wards, patient-nurse ratio is low, and there is low compliance with preventive measures. In an Egyptian respiratory ICU (in Cairo) VAP rate were 73.4 per 1000 ventilation days by CDC definition.

Third: There were higher device associated infection rates and higher device-utilization ratios in major teaching hospitals (those hospitals with an important role in teaching programs and with clinical clerkships) than from all other hospitals. These
differences may result from differences in case mix (including differences in numbers of patients with severe trauma or with organ transplantation), in staffing, or in procedures. Fourth: most of the surveillance system record the first VAP episodes or utilize the more rigid CDC criteria for diagnosis of second VAP attack by complete resolution of initial infections. Therefore, the high VAP rate per 1000 ventilator days in both the pre and the post-intervention phases showed the compelling need that initiated the present study and still demanding for further VAP intervention Campaigns.

The major limitation of this study was the long time lag between the pre- and post-intervention phase (51 months) which might contributed to the resistance pattern of organisms prevailing. Although, this might be partly attributed to the lack of resources for program implementation, this might have attenuated the effect of the educational program. The second limitation of this study was that the personnel collecting the compliance to VAP preventive practices were different in both phases. In addition the observation of VAP preventive practices was only done in the morning shift in both the pre and post-intervention phases of both units.

The third limitation that both pre and post-intervention phases were observational non-randomized study (ICU staff and patients were non-blinded to the intervention). This raised the possibility of factors that have occurred which accounted for these results. For these reasons, we evaluated confounding factors which are considered the risk factors for VAP and used it for risk stratification. However the double blinded randomized trial is not applicable for the educational program because of failure to segregate its effect from study and control groups and also randomization of patients to receive or not to receive good care that has been recognized by IHI and Joint Commission on Accreditation of Healthcare Organizations’ (JCAHO) may be unethical. Therefore educational program can only be applied through pre and post-intervention observation study as in all previous studies. Other factors like seasonal variation has been excluded as pre and post-intervention phase has been performed in the same months (both from September till May).

The fourth limitation; that this was a single medical center study, so the results cannot be generalized to other hospitals. However the quite similar results from several studies, suggests that this intervention might be applicable for other resource limited facilities, especially in developing countries. For this reasons, we explained our setting in details so that it can be compared to other facilities.

The fifth limitation of our study was its susceptibility to a number of biases. The clinical diagnosis by the modified ACCP criteria had limited specificity and the possibility of misclassification bias cannot be ruled out. However the same observer and diagnostic criteria were used in both the pre and post-intervention study groups which might have attenuated the effect of any misclassification bias. Also the same personnel performed all patients’ data collection in the pre and post-intervention study group. Thus observer bias might have occurred, although discussion of all suspected VAP cases have been done with surveillance team. Similarly the health-care worker was informed of surveillance and might have haw-throne effect (observer effect).

In spite of the importance of top management support and full availability of supplies, VAP staff education was still effective in reducing VAP through tailoring of educational and procedural tools. As in relation to our initial failure to reduce VAP rate as with Hawe et al, Papadimos et al, Bigham et al, and Cocanour et al it is suggested that active implementation strategies and multi-module program increase the success of VAP prevention programs.

REFERENCES
7. Torres A, Ewig S, Lode H, Carlet J. Defining, treating and


doi: 10.1056/NEJM199902253400807


40. Young MP, Manning HL, Wilson DL, et al. Ventilation of patients with acute lung injury and acute respiratory distress syn-


