

## **$\alpha$ -2-Antiplasmin : A Fibrinolytic Peptide, could be a Potential Biomarker for Diagnosis of High Altitude Induced Pulmonary Hypertension**

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### **ABSTRACT**

Prolonged exposure to high altitude is known to cause pulmonary vascular remodeling and pulmonary hypertension. Despite the advancements in diagnostics of pulmonary hypertension (PH), a potential biomarker for early diagnosis of PH progression is still lacking. High altitude induced pulmonary hypertension (HAPH) is great concern in mountain regions across the world. Early diagnosis and monitoring the disease progression are critical for therapeutic decisions. A fibrinolytic peptide, alpha-2-antiplasmin ( $\alpha$ 2AP) is known to cause tissue and vascular remodeling. Recently, elevated levels of  $\alpha$ 2AP were reported in different forms of PH; however its role in high altitude induced pulmonary hypertension is not yet known. To assess whether  $\alpha$ -2AP can be used as diagnostic marker for HAPH, a cross sectional study was carried out on 100 healthy male Indian army soldiers who were posted to high altitude (14,800 ft to 18,700 ft) for a prolonged period of 6 to 8 months. We used 2D Echocardiography to screen the pulmonary arterial systolic pressure (PASP) and semi-quantitatively ELISA method for estimation of  $\alpha$ -2AP in plasma. We found 34 out of 100 subjects have elevated PASP upon immediate screening after de-induction to moderate altitude of 10,000 ft. Age matched 17 subjects with elevated PASP were assessed for plasma levels of  $\alpha$ -2AP in comparison to subjects with normal PASP, but posted to same altitude for same duration of time. Here for the first time we demonstrated an increase in plasma level of  $\alpha$ -2AP was positively correlated with hypoxia induced elevated pulmonary arterial pressure. However, additional comprehensive investigations are warranted with large sample size to confirm our findings and explore the use of  $\alpha$ 2AP as potential diagnostic marker for monitoring progression of HAPH.

**Keywords:** High altitude; Pulmonary hypertension; Alpha-2-antiplasmin; Fibrinolysis

### **1. INTRODUCTION**

High altitude pulmonary hypertension (HAPH), is a debilitating disease which primarily affects the people who live in high altitude thus a major public health concern in mountain regions throughout the world<sup>1,2</sup>. Around 140 million people permanently reside at high altitudes and more than 40 million people visit these areas for recreational and other reasons<sup>2</sup>. Indeed, some lowland individuals were also reported to develop HAPH due to prolong exposure high altitude<sup>2,3</sup>. HAPH has been classified in third group under WHO pulmonary hypertension (PH) classification and defined by an increase in mean pulmonary arterial pressure ( $P_{pa}$ ) >25 mmHg at rest or > 30 mmHg at exercise<sup>1</sup>. Though pathophysiology of HAPH is not completely understood, but largely believed to be associated with excessive polycythemia, disproportionate pulmonary vascular remodelling, and imbalance in hemostasis pathways<sup>2</sup>. Singh<sup>4,5</sup>, *et al.* first reported in 1965 and 1972 that Indian soldiers posted at high altitude (12000 ft - 18000 ft) for a period more than 5 months develop pulmonary hypertension and further suggested that blood coagulation changes due to prolong exposure to high altitude predispose to pulmonary hypertension. It was later speculated that high altitude

induced blood coagulation changes accompanied by decrease in activity of fibrinolytic peptides leads to development of thrombi and fibrin deposition in pulmonary arteries<sup>6</sup>. However the molecular mechanisms leading to fibrin deposition and subsequent changes in pulmonary circulation are not yet understood completely.

Plasmin is the key fibrinolytic peptide which cleaves fibrin/collagen, disbands thrombi and degrades extracellular matrix (ECM)<sup>7</sup>. Plasmin has also been reported to activate matrix metalloproteases (MMPs), which play central role in regulating ECM turnover<sup>7,8</sup>. However, the activity of plasmin is partly regulated by plasminogen activator inhibitor (PAI-1) and largely regulated by alpha-2-antiplasmin ( $\alpha$ 2AP)<sup>7,8</sup>. The  $\alpha$ 2AP, a 67 kd serine protease inhibitor (serpin), was reported to be synthesized in liver, kidney and brain<sup>8</sup>. Lately,  $\alpha$ 2AP has gained enormous interest for its role in pathogenesis of several cardiovascular diseases<sup>9</sup>. An elevated level of  $\alpha$ 2AP was first reported in PH associated with scleroderma patients and later, similar trend was reported in other classes of PH such as chronic thromboembolic pulmonary hypertension and idiopathic pulmonary arterial hypertension<sup>9</sup>. The key characteristic of PH is pulmonary vascular remodeling, marked by excessive pulmonary vascular smooth muscle cell (PVSMC) proliferation, endothelial medial transformation (EMT) and adventitial differentiation<sup>10,11</sup>. Kanno<sup>12</sup>, reported that  $\alpha$ 2AP regulates EMT

of myofibroblasts, differentiation of tissue-resident fibroblasts and bone marrow-derived mesenchymal stem cells (MSCs). Further,  $\alpha$ 2AP has been reported to be upregulated in thoracic arteries and femoral arteries subsequent to hormonal or injurious stimuli<sup>13,14</sup>. Substantiating the clinical observations, experimental studies in mice deficient in  $\alpha$ 2AP have shown decreased collagen deposition and diminished myofibroblasts transdifferentiation in vascular injury model<sup>12</sup>. Additionally, it was reported that  $\alpha$ 2AP deficient mice have increased plasmin activity which causes over-release of vascular endothelial growth factor (VEGF) and consequently improved endothelial repair to maintain vascular patency<sup>15</sup>. Clearly, these studies signify the physiological role of  $\alpha$ 2AP in vascular remodeling. Lately, subjects suffering from obstructive sleep apnea have also been shown with increased  $\alpha$ 2AP in plasma<sup>16</sup>. All these studies intrigued us to assess the effects of hypobaric hypoxia environment on alpha-2-antiplasmin expression and to evaluate its correlation to pulmonary hemodynamics.

## 2. METHODS

### 2.1 Ethical Clearance

The study was approved by the institutional human ethical committee of Defense Institute of Physiology & Allied Sciences (DIPAS) in line with ICMR guidelines which are in accordance with the latest version of the Declaration of Helsinki. All volunteers were briefed about the study and written informed consent was obtained from all participants in the study. Only fresh inductees within age group of 20 yr to 45 yr and with minimum 6 m duration of stay at high altitude were included in the study. Participants with previous history of cardiac, cerebrovascular, chronic respiratory, renal or hepatic diseases, AMS, HAPE or HACE were not included in the study.

### 2.2 Study Subjects and Protocol

Subjects of the current study were classified on the basis of clinical characteristics and echocardiography data. Age matched 17 subjects, presented with an elevation in pulmonary arterial systolic pressure (PASP) were compared against the 17 control subjects with normal PASP from the same cohort, exposed to similar heights and same duration of stay. The present cross-sectional study involved 100 Indian soldiers aged between 21 yr to 45 yr (mean age; 31 yr), posted at high altitude heights ranging 14,800 ft to 18,700 ft in Western Himalayas for an average of  $7.25 \pm 0.8$  months and de-inducted by foot to field hospital in Drass, at an altitude of 10,000 ft from sea level, Jammu & Kashmir, India. Out of 100 soldiers 34 had elevated PASP; out of 34 we chose 17 age matched subjects with elevated PASP (e-PAP) and 17 age matched subjects from same cohort with normal PASP (HA-C) for semi-quantitative ELISA studies to estimate relative expression levels of  $\alpha$ 2AP between comparing groups. Continuous wave Doppler was used to measure the peak velocity ( $v$ ) of the tricuspid regurgitant (TR) jet and right atrial pressure (RAP) was estimated by evaluating the inferior vena cava (IVC) size and collapsibility with respiration. The PASP was assessed using the modified Bernoulli equation ( $4v^2 +$  estimated RA pressure) and mean arterial pressure was calculated using following formula ( $mPAP = 0.61 * PASP + 2$  mmHg)<sup>17</sup>.

### 2.3 Estimation of $\alpha$ 2AP by Semi-quantitative ELISA

Blood was collected in a BD Vacutainer PP- K3 EDTA (BD Biosciences, India), kept on ice and centrifuged within 30 min. Plasma was separated by centrifugation at 1500 g for 5 min and stored at  $-80$  °C until assayed. Normalization of samples for the protein levels was done by Bradford method. To perform enzyme linked immunosorbant assay (ELISA), 50  $\mu$ g of plasma was incubated with an equal volume of coating buffer [0.5 M carbonate buffer (pH 9.6)] in a 96 well assay plate overnight at 4 °C. Following day, 5 per cent bovine serum albumin was used to block any non-specific binding for 30 minutes at room temperature. The sample wells were then washed with PBS containing 0.05 per cent Tween20, followed by 4 hr incubation with  $\alpha$ 2AP primary antibody in blocking buffer (1:250). The samples were next washed and incubated with diluted goat anti-rabbit IgG-HRP conjugated secondary antibody (1:1000) in the same buffer for 2 hr. After being washed, the samples were incubated with p-nitrophenyl phosphate (1 mg/mL) in carbonate buffer containing 10 mM MgCl<sub>2</sub>. The reaction was stopped by adding 50  $\mu$ L of 1 M NaOH and the development of color was assessed at 450 nm<sup>18</sup>. The results of relative  $\alpha$ 2AP levels were assessed and statistically evaluated using Prism5 (GraphPad).

### 2.4 Data Analysis

Data are represented as mean  $\pm$  SEM. The comparison in expression of  $\alpha$ 2AP between e-PAP and HA-C groups was performed by student's t-test was used and a value of  $p < 0.001$  was considered significant assessed by Prism5 (GraphPad).

## 3. RESULTS

### 3.1 Study Participant Characteristics

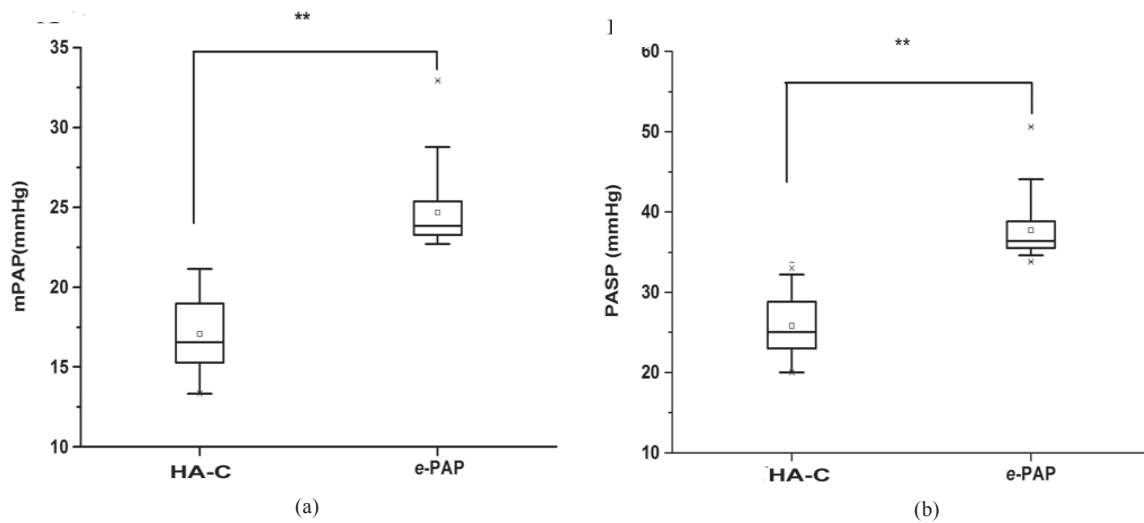
Table 1 presents the physiological characteristics of the participants in study.

#### 3.1.1 Pulmonary Hemodynamics in Subjects after Prolonged Stay at High Altitude

66 out of 100 subjects exposed to a mean altitude of  $16789 \pm 100$  ft for a period of  $7.25 \pm 0.8$  months showed a PASP of  $25.8 \pm 0.5$  mmHg and mPAP of  $17.7 \pm 0.3$  mmHg, respectively immediately after de-induction to moderate altitude (Drass  $\sim$  10,000 ft); this group was designated as HA-C. Conversely, remaining 34 subjects of the same cohort,

**Table 1. Physiological characteristics of study population**

Variables	Mean $\pm$ SEM
Age	31 $\pm$ 0.6
Height (cm)	170.4 $\pm$ 1.6
Body mass index (kg/m <sup>2</sup> )	19.6 $\pm$ 1.14
Average altitude of residence (ft)	16789 $\pm$ 100
Systolic blood pressure (mm Hg)	115 $\pm$ 1.2
Diastolic blood pressure (mm Hg)	81 $\pm$ 1
Heart rate (beats/min)	68 $\pm$ 1
Oxygen saturation ( per cent)	95 $\pm$ 0.2



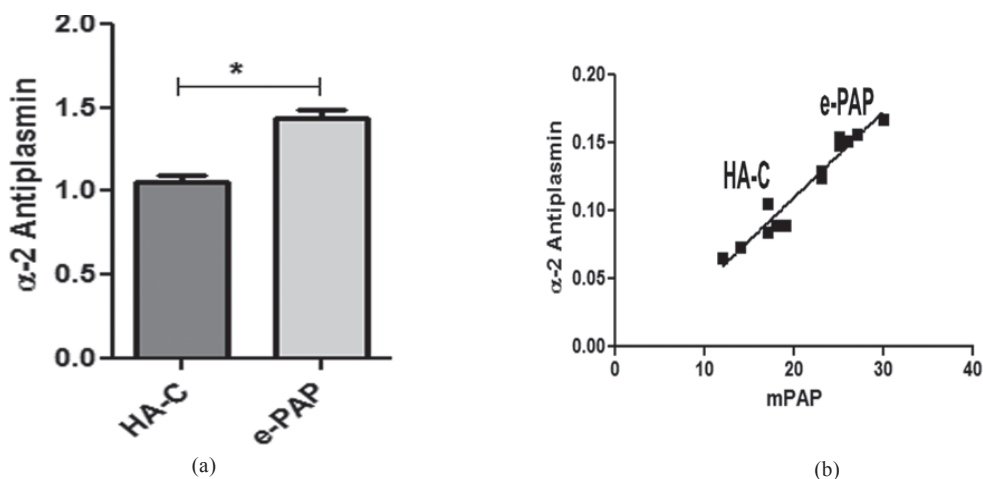
**Figure 1.** Hemodynamic assessment of subjects exposed to high altitude through graphical representation of comparatives: (a) mean pulmonary arterial pressure (mPAP) between HA-C and e-PAP groups. (b) pulmonary arterial systolic pressure (PASP) between HA-C and e-PAP groups (distribution and median values were also shown along with means). Statistical significance of means was determined by the Mann-Whitney test (A&B). \* $p < 0.05$  was considered significant.

exposed to similar heights for same duration of time, showed a significant ( $p < 0.05$ ) elevation in PASP ( $37.7 \pm 0.6$  mmHg) and mPAP ( $24.9 \pm 0.3$  mmHg) as compared to HA-C group; this group was designated as e-PAP as shown in Fig. 1. Out of 34 subjects with e-PAP, only 5 subjects were found to be falling under diagnostic criteria of mild PH with elevated PASP ( $40.56 \pm 0.4$  mmHg) and mPAP ( $26.7 \pm 0.2$  mmHg). Nevertheless, all the subjects with e-PAP were reported to be asymptomatic and showed no clinical signs of PH such as dyspnoea and chest pain during exertion.

### 3.2 $\alpha$ 2AP Levels were Significantly Increased in Plasma of e-PAP Subjects

Seventeen subjects in control group (HA-C) and seventeen subjects in e-PAP group matched with age ( $\sim 30$

yr), altitude of residence ( $\sim 16780$  ft) and duration of stay ( $\sim 7.25 \pm 0.8$  months) were randomly picked and processed for comparing  $\alpha$ 2AP levels in plasma. Here, we have reported for the first time that  $\alpha$ 2AP levels were significantly increased in subjects with elevated pulmonary arterial pressure (e-PAP) as compared to subjects with no evident change in pulmonary pressure as shown in Fig. 2. We observed 0.5 fold increase in  $\alpha$ 2AP levels of e-PAP plasma samples as compared to as compared to control. Furthermore, we performed a relative analysis of increased  $\alpha$ 2AP and elevated pulmonary pressure in each subject and plotted relative linear regression graph as shown in Fig. 2(b). We observed subjects with clinical pulmonary hypertension ( $>25$  mmHg mPAP;  $n=5$ ) had significantly ( $p < 0.001$ ) high levels of  $\alpha$ 2AP as compared to HA-C group.



**Figure 2.**  $\alpha$ 2AP is upregulated in subjects with elevated pressure (a) 34 out of 100 subjects had elevated pulmonary pressure. Plasma samples of 17 subjects from control (HA-C) subjects and 17 from elevated pulmonary pressure (e-PAP) were processed for ELISA. Representative graph above shows  $\alpha$ 2AP expression levels increased significantly in e-PAP subjects as compared to HA-C ( $p < 0.01$ ) assessed by Prism5 (GraphPad) and (b) Linear regression plot of  $\alpha$ 2AP vs mPAP showed a significant increase in  $\alpha$ 2AP levels in each individual with elevated mPAP plotted by Prism5 (GraphPad).

### 3.3 Discussion

The present study demonstrated that the prolonged stay at high altitude markedly increased the pulmonary arterial pressure in significant number of people. Additionally, our study also showed for the first time an increase in  $\alpha$ 2AP is positively correlated with elevated pulmonary pressure.

Early diagnosis of pulmonary hypertension still remains a challenge. Although, right heart catheterization and echocardiography have revolutionized the field of PH diagnosis but these techniques are laborious and require a highly qualified expert and facility to perform. Moreover, various serological/circulating markers also have been proposed for the diagnosis of PH. These markers are generally related to ventricle wall stress, endothelial dysfunction or oxidative stress. Most acclaimed marker for PH diagnosis currently is atrial natriuretic factor and brain natriuretic factor, which are released post ventricular wall stress<sup>19-21</sup>. In recent years N terminal pro-BNP (NT-proBNP) earned more interest over BNP as a markers for its prolonged stability<sup>19,20</sup>. However, the release of BNP/NT pro-BNP is triggered once the ventricular walls are under stress, which is a very late stage of PH, making it impracticable for the early detection of PAH<sup>19,20</sup>. Similarly endothelin-1(ET1), uric acid, troponin T, nitric oxide (NO) levels have been also proclaimed to be diagnostic markers of PH, however these are highly influenced by ethnicity, age and sex lowering the diagnostic interpretation<sup>19-21</sup>.

HAPH has been being reportedly associated with increased vascular wall thickness, polycythemia and altered hemostatic balance<sup>2</sup>. Further it has been demonstrated that high altitude induced an increase in pro-coagulation state was associated with decrease in fibrinolytic activity, owing to increased antiplasmin peptides<sup>6</sup>. Conversely, some studies have demonstrated that high altitude exposure decrease the expression of coagulation factors and increase the fibrinolytic peptides<sup>22</sup>. Supporting the earlier notion, experimental animal studies have also demonstrated an increase in levels of coagulatory factors in animals exposed to hypobaric hypoxic<sup>23,24</sup>. High altitude living has been reported to cause pulmonary hypertension and however the pathophysiological mechanisms not yet understood completely. In the current study, we observed that prolonged exposure of sea level troops to high altitude causes marked elevation of PAP. Here, for the first time, we have reported a significant increase in circulating  $\alpha$ 2AP levels in subjects with elevated PAP as compared to subjects with normal PAP. Interestingly, oxygen saturation returned to almost 95 per cent in both comparative groups upon de-induction from extreme altitude to moderate altitude (Drass ~10,000 ft), however rise in PAP persisted only in few subjects, indicating possible structural and functional changes in pulmonary circulation in these subjects. Similar to our findings, Singh<sup>4,5</sup>, *et al.* reported significantly increase in PAP in sea level troops posted to extreme altitude.

Mostly, Indian troops are posted to high altitude (3500 to 5500 m) for a total period of 2 years including extreme altitude (> 5500 m) employment for a period of 3 months. Thereby indicating the prolonged exposure to high altitude makes them highly vulnerable to development of PAH. In current

study we noticed that 34 per cent of total study volunteers had an elevated PAP, out of which only 5 per cent of them were falling under criteria of PH. Knowing that the increase in PAP affects the exercise performance and predispose them to high altitude maladies, studying the effect of prolonged stay at high altitude on pulmonary circulation rather become imperative for early diagnosis of HAPH. Our preliminary finding strongly speculates that  $\alpha$ 2AP could be a novel biomarker for early diagnosis HAPH. A positive correlation demonstrated between  $\alpha$ 2AP and mPAP in current study further merits a comprehensive study on role of  $\alpha$ 2AP in pathophysiology of HAPH.

### 4. CONCLUSION

Increased expression levels of  $\alpha$ 2AP are associated with elevated pulmonary arterial pressure signifying potential use of  $\alpha$ 2AP as an early diagnostic marker for PH detection.

### 5. LIMITATIONS OF STUDY

In this study we have shown that  $\alpha$ 2AP is positively correlated to elevated pulmonary arterial pressure in subjects exposed to high altitude for prolonged periods. Though, the study provides a new paradigm for the diagnosis and therapeutic target of HAPH, the current findings needs to be further validated in larger study group. In addition since the study was cross sectional so we didn't record the respective sea level, pre/post- induction, mPAP and  $\alpha$ 2AP.

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#### CONFLICT OF INTEREST

None of the authors have any conflict of interest. All authors reviewed and approved the final draft of the manuscript.

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In the current study, he was involved in execution of experimental studies, data collection, data analysis and manuscript writing.

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In the current study, he was involved in conceptualisation and designing the study, designing, data interpretation and manuscript writing.