

# Elevated blood plasma levels of epinephrine, norepinephrine, tyrosine hydroxylase, TGF $\beta$ I, and TNF $\alpha$ associated with high-altitude pulmonary edema in an Indian population

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**Abstract:** Biomarkers are essential to unravel the locked pathophysiology of any disease. This study investigated the role of biomarkers and their interactions with each other and with the clinical parameters to study the physiology of high-altitude pulmonary edema (HAPE) in HAPE-patients (HAPE-p) against adapted highlanders (HLs) and healthy sojourners, HAPE-controls (HAPE-c). For this, seven circulatory biomarkers, namely, epinephrine, norepinephrine, tyrosine hydroxylase, transforming growth factor beta 1, tumor necrosis factor alpha (TNF $\alpha$ ), platelet-derived growth factor beta beta, and C-reactive protein (CRP), were measured in blood plasma of the three study groups. All the subjects were recruited at ~3,500 m, and clinical features such as arterial oxygen saturation (SaO<sub>2</sub>), body mass index, and mean arterial pressure were measured. Increased levels of epinephrine, norepinephrine, tyrosine hydroxylase, transforming growth factor-beta 1, and TNF $\alpha$  were observed in HAPE-p against the healthy groups, HAPE-c, and HLs ( $P < 0.0001$ ). CRP levels were decreased in HAPE-p against HAPE-c and HLs ( $P < 0.0001$ ). There was no significant difference or very marginal difference in the levels of these biomarkers in HAPE-c and HLs ( $P > 0.01$ ). Correlation analysis revealed a negative correlation between epinephrine and norepinephrine ( $P = 4.6E-06$ ) in HAPE-p and positive correlation in HAPE-c ( $P = 0.004$ ) and HLs ( $P = 9.78E-07$ ). A positive correlation was observed between TNF $\alpha$  and CRP ( $P = 0.004$ ) in HAPE-p and a negative correlation in HAPE-c ( $P = 4.6E-06$ ). SaO<sub>2</sub> correlated negatively with platelet-derived growth factor beta beta (HAPE-p;  $P = 0.05$ ), norepinephrine ( $P = 0.01$ ), and TNF $\alpha$  ( $P = 0.005$ ) and positively with CRP (HAPE-c;  $P = 0.02$ ) and norepinephrine (HLs;  $P = 0.04$ ). Body mass index correlated negatively with epinephrine (HAPE-p;  $P = 0.001$ ) and positively with norepinephrine and tyrosine hydroxylase in HAPE-c ( $P < 0.05$ ). Mean arterial pressure correlated positively with TNF $\alpha$  in HAPE-p and norepinephrine in HLs ( $P < 0.05$ ). Receiver operating characteristic curve analysis yielded a positive predictive value for these biomarkers with HAPE (area under the curve  $> 0.70$ ,  $P < 0.05$ ). The results clearly suggest that increased plasma levels of these circulatory biomarkers associated with HAPE.

**Keywords:** adaptation, biomarkers, correlation, HAPE, high-altitude, sympathetic nervous system

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## Introduction

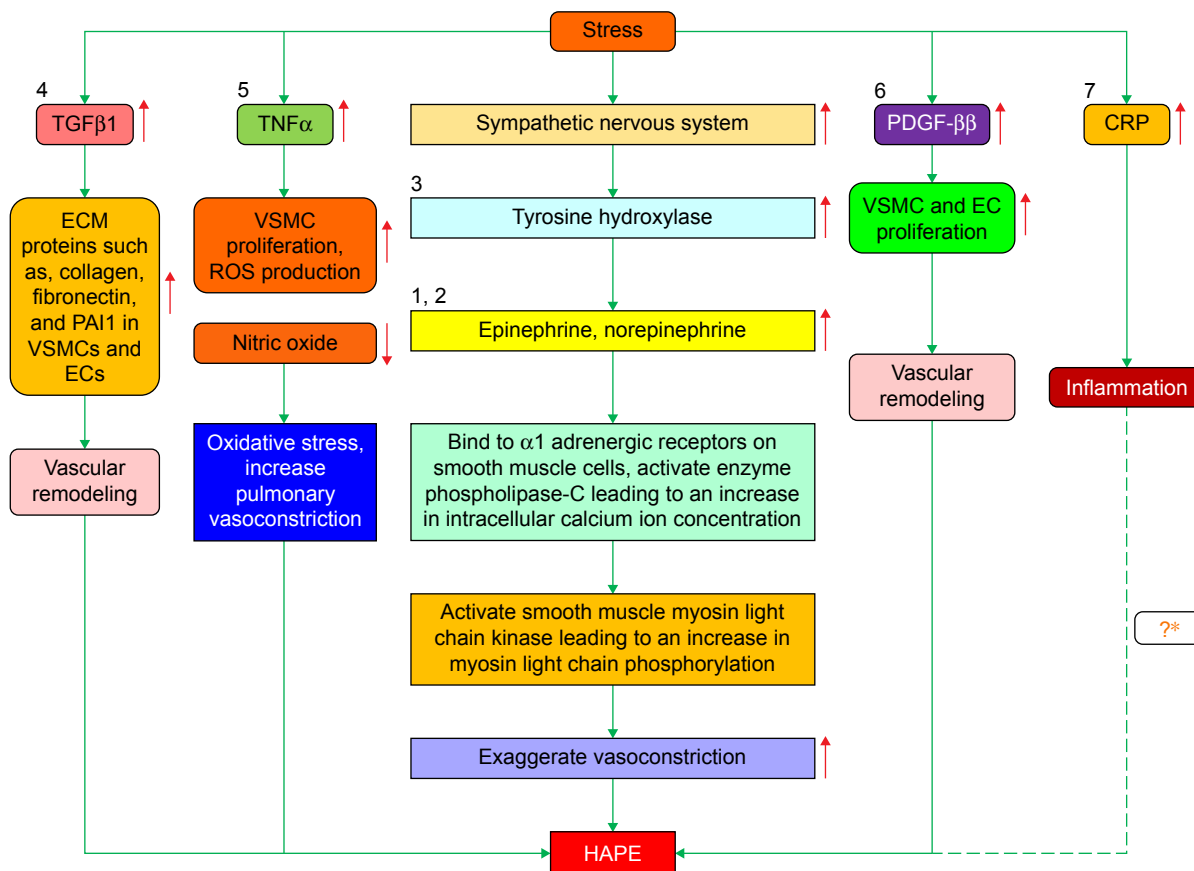
Extreme environmental conditions at high altitude (HA) cause a number of disorders in sojourners, while at the same time, permanent residents of the region remain in good physical shape. In this study, our primary concern has been associated with a rare, fatal (if not treated), and morbid respiratory illness, high-altitude pulmonary edema (HAPE).

HAPE occurs in unacclimatized sojourners upon rapid ascent to HA regions. It is imperative to emphasize here that only a small population of sojourners gets afflicted with the disorder, while a large fraction remain healthy. HAPE is associated with abnormal build up of fluid in lungs, leading to signs and symptoms such as dyspnea at rest, lethargy, tachycardia, and congestion.<sup>1-3</sup> Since estimating circulatory biomarkers has always been an established mechanism to understand the pathophysiology of any disease,<sup>4,5</sup> in this study, we have studied seven circulatory biomarkers, namely, epinephrine, norepinephrine, tyrosine hydroxylase (TH), transforming growth factor beta 1 (TGFβ1), tumor necrosis factor alpha (TNFα), platelet-derived growth factor beta beta (PDGF-ββ), and C-reactive protein (CRP). The above-mentioned seven biomarkers are known to be related to the processes of exaggerated vasoconstriction and smooth muscle proliferation that are hallmark characteristics of HAPE (Figure 1).

Hypobaric hypoxic environment at HA activates sympathetic nervous system.<sup>6-10</sup> Stress-activated sympathetic nervous

system releases epinephrine and norepinephrine through adrenal medulla via the enzyme TH. Epinephrine and norepinephrine activate G<sub>q/11</sub>-coupled alpha 1-adrenergic receptors on the pulmonary smooth muscle cells, leading to an increase in intracellular Ca<sup>2+</sup> concentrations (Figure 1).<sup>11-13</sup> Increased calcium ion concentration activates smooth muscle cell-specific myosin light chain kinase, which, in turn, phosphorylates myosin light chain at serine residue 19, inducing the formation of actin–myosin cross-bridge formation, thus causing exaggerated vasoconstriction and leading to HAPE (Figure 1).<sup>14,15</sup>

Among the other molecules, TGFβ1, TNFα, PDGF-ββ, and CRP are the circulatory biomarkers that are induced under stress conditions.<sup>16-19</sup> To the best of our knowledge, blood plasma levels of TGFβ1, TNFα, PDGF-ββ, and CRP have not been reported in such a large sample size and study module among the three study groups, namely, HAPE-patients (HAPE-p), HAPE-controls (HAPE-c), and permanent residents of HAs, that is, highlanders (HLs). TGFβ1, a regulator of collagen synthesis and vascular



**Figure 1** Pathway specifying the role of the studied biomarkers (1–7) in the progression of HAPE.

**Notes:** Upward red arrows: the process or the molecule is upregulated. Downward red arrow: the process or the molecule is downregulated. \*Dashed green arrow indicates it is not yet entirely known whether or not that inflammation occurs in HAPE.

**Abbreviations:** HAPE, high-altitude pulmonary edema; TGFβ1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; PDGF-ββ, platelet-derived growth factor beta beta; CRP, C-reactive protein; ECM, extracellular matrix; PAI1, plasminogen activator inhibitor 1; VSMCs, vascular smooth muscle cells; ROS, reactive oxygen species; ECs, endothelial cells.

remodeling in fibroblasts,<sup>20–22</sup> and PDGF- $\beta\beta$ , a pro-proliferative growth factor,<sup>23–25</sup> are known to cause increased smooth muscle proliferation and thus vascular remodeling, a key process associated with HAPE (Figure 1). Since inflammation in HAPE is still a subject of contradictions, two major inflammatory mediators, that is, TNF $\alpha$ , a pleiotropic proinflammatory cytokine,<sup>26–28</sup> and CRP, a proinflammatory marker,<sup>29,30</sup> were also studied (Figure 1).

Therefore, this study aimed to determine the biochemical features associated with HAPE. For this, three-study group formula was adopted, that is, biochemical parameters measured in HAPE-p were compared with the two healthy groups. One healthy group was constituted of lowland population akin to the HAPE-p, that is, HAPE-c, and the second healthy group was constituted of the highland population, that is, permanent residents of the HA regions, the HLs. Given that individual molecule cannot run the whole disease pathophysiology, henceforth, a correlation study among the biomarkers and between various clinical parameters, such as arterial oxygen saturation (SaO<sub>2</sub>), body mass index (BMI), and mean arterial pressure (MAP), and biomarkers was also performed. The selected biomarkers were studied as all the seven were associated with the hallmark processes of HAPE, exaggerated vasoconstriction and smooth muscle cell proliferation, and their role in the physiology of HAPE per se has not been studied in great details (Figure 1).

## Materials and methods

### Ethics statement

The study protocol was approved by the human ethical committees of both the CSIR-Institute of Genomics and Integrative Biology, Delhi, and the Sonam Norboo Memorial Hospital, Leh, Ladakh. All the subjects were recruited by Sonam Norboo Memorial Hospital, Leh, Ladakh. Written informed consent was obtained from each subject for participation in the study.

### Study groups

The study comprises three well-defined groups: 1) HAPE-p: sojourners who acquired HAPE upon ascent to HA (~3,500 m) on first exposure, 2) HAPE-c: sojourners who did not acquire HAPE upon ascent to HA (~3,500 m) under similar conditions as experienced by HAPE-p, 3) HLs: healthy natives of the HA region, that is, residing for generations at HA. Whole sample collection was performed in Leh, Ladakh (HA), and experimental analysis was performed in our base laboratory at Delhi.

The number of subjects incorporated per group was varied owing to the difference in timing and volume of

samples being collected and simultaneous consumption in various assays. The actual number of samples incorporated per group has been mentioned in Table 1.

### Selection criteria

1) A detailed medical examination was performed, and the clinical findings that are mandatory for HAPE were recorded in an exhaustive questionnaire that was administered to the participants. The diagnosis of HAPE was based on chest radiographic infiltrates being consistent with pulmonary edema. The other clinical symptoms included hypoxemia, cough, and dyspnea at rest, breathlessness, presence of pulmonary rales, absence of any infection, reduced exercise performance, and cyanosis. The clinical parameters, for example, age, sex, BMI, blood pressure, and SaO<sub>2</sub>, were also measured. Lake Louise scoring was applied to rule out any symptoms of acute mountain sickness among HAPE-p and HAPE-c. 2) HAPE-p and HAPE-c were ethnically matched. 3) Both the HAPE-c and the HLs were declared healthy and fit, devoid of any disorder. 4) All the study subjects are permanent residents of India. 5) Only subjects within 15–50 years of age were recruited for the study. Of note, HAPE-p were the individuals who had developed HAPE upon their first visit to the HA region.

### Sample collection

Ten milliliters of venous blood was drawn in acid-citrate-dextrose anticoagulant tubes (~3,500 m) with subjects in supine position. The plasma was collected after centrifuging the tubes at 1,500 rpm for 5 minutes and was stored at –80°C until analysis. Patient's blood was collected once HAPE was diagnosed but prior to the beginning of any treatment. Blood from the subjects of the two healthy groups, that is, HAPE-c and HLs, was drawn following overnight fasting. The samples were collected, aliquoted, and preserved at –80°C over a period of years. The experiments and the subsequent analysis were performed in the year 2014. The subjects ascended from the base (Delhi ~200 m) to the HA region (Leh ~3,500 m) by air in an airplane. HAPE was diagnosed in the subjects within 1 week of their arrival at the HA region.

### Biomarker estimation

Commercially available enzyme-linked immunosorbent assay kits were used to measure the blood plasma epinephrine (BA E-5100; LDN, Nordhorn, Germany), norepinephrine (BA E-5200; LDN), TH (E91438Hu; Usn Life Science Inc., Houston, TX, USA), TGF $\beta$ 1 (E90124Hu; Usn Life Science Inc.), TNF $\alpha$  (E90133Hu; Usn Life Science Inc.), PDGF- $\beta\beta$  (E90633Hu; Usn Life Science Inc.), and CRP (E90821Hu; Usn Life Science Inc.). The measurements were taken at

Table 1 Plasma levels of the seven biomarkers

Biomarker	Mean ± SEM (n)		HAPE-p versus HAPE-c			HAPE-p versus HLs			HLs versus HAPE-c		
	HAPE-c	HAPE-p	B	t	P-value	B	t	P-value	B	t	P-value
Epi (pg/mL)	46.17±1.29 (203)	61.69±1.58 (196)	16.92	8.20	3.31E-15*	15.21	5.35	1.75E-07*	1.02	0.37	0.71
Norepi (pg/mL)	81.46±2.11 (203)	109.01±1.35 (204)	25.71	10.15	1.05E-21*	20.99	7.99	2.59E-14*	4.22	1.13	0.26
TH (pg/mL)	3.59±0.33 (48)	8.12±0.69 (54)	4.47	5.59	2.04E-07*	4.16	4.70	9.15E-06*	0.26	0.51	0.61
TGFβ1 (pg/mL)	3,200.56±203.98 (49)	4,528.94±259.19 (53)	1,374.31	4.09	8.97E-05*	2,506.73	7.07	3.33E-10*	-1,139.79	-3.85	0.0002*
TNFα (pg/mL)	53.95±4.27 (49)	119.02±7.18 (53)	64.22	7.50	2.94E-11*	76.58	8.20	1.71E-12*	-13.30	-2.09	0.04*
PDGF-ββ (pg/mL)	2,687.54±18.34 (49)	2,619.67±39.12 (53)	-77.52	-1.75	0.08	138.23	1.58	0.12	-209.91	-2.55	0.01*
CRP (ng/mL)	4.39±0.38 (49)	1.00±0.08 (53)	-3.37	-8.72	7.24E-14*	-5.11	-13.15	1.13E-22*	1.77	2.97	0.004*

Notes: Univariate analysis of covariance was performed for the analysis. SPSS Version 16 software has been used. P-values are adjusted for age and sex. Data are presented as mean ± SEM. B, regression coefficient; t, t-statistic. \*Cutoff for significance is P<0.05.

Abbreviations: SEM, standard error of the mean; n, number of samples; HAPE, high-altitude pulmonary edema; HAPE-p, HAPE-patients; HAPE-c, HAPE-controls; HLs, highlanders; Epi, epinephrine; Norepi, norepinephrine; TH, tyrosine hydroxylase; TGFβ1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; PDGF-ββ, platelet-derived growth factor beta beta; CRP, C-reactive protein.

appropriate wavelengths on a Benchmark Plus Microplate Spectrophotometer System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Manufacturer’s protocol was adhered for the estimations.

## Statistical analysis

### Biomarker levels

All the data were expressed as mean ± standard error of the mean. The levels of the biomarkers were analyzed using the statistical regression model, univariate analysis of covariance on SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant. P-values were adjusted for age and sex as confounding factors to nullify their effects. HAPE-c were the reference group in HAPE-p versus HAPE-c and HLs versus HAPE-c analysis. HLs were the reference group in HAPE-p versus HLs analysis.

### Correlation analysis

A partial correlation model was fitted for the correlation analysis between 1) the studied biomarkers, 2) SaO<sub>2</sub> and biomarkers, 3) BMI and biomarkers, and 4) MAP and biomarkers. Whole correlation analysis was performed on SPSS 16.0 software. All the graphs were constructed using MS excel. A P-value of <0.05 was considered statistically significant for biomarker evaluation and correlation analysis between 1) SaO<sub>2</sub> and biomarkers, 2) BMI and biomarkers, and 3) MAP and biomarkers. Since the sample size overlapping per group for inter-biomarker correlation analysis was relatively small, in order to keep the consistency, coherency, and stringency of the results, the statistical outcomes/results were corrected for multiple comparisons using Bonferroni test leading to drop in significance cutoff to 0.006–0.01 from 0.05. As the total number of biomarkers studied is seven, 0.05/7=0.007–0.01 is the new cutoff. This has reduced the chances of any false-positive outcome being incorporated in the results. A two-tailed P-value of <0.01 was considered statistically significant for inter-biomarker correlations in Table 2. All the P-values were adjusted for age and sex as confounders to nullify their effects.

### Receiver operating characteristic curve

Receiver operating characteristic (ROC) curve analysis was performed on biomarker levels using SPSS 16.0 software. P<0.05 was considered statistically significant. Area under the curve (AUC) >0.70 was considered a positive test for prediction value of a biomarker. Distribution assumption was nonparametric.

**Table 2** Correlation analysis between biomarkers in the three study groups

Biomarker	Epi	Norepi	TH	TGFβ1	TNFα	PDGF-ββ	CRP
Epi	I			HAPE-p			
Norepi	$r=-0.61$ $P=4.6E-06^*$	I					
TH	$r=-0.04$ $P>0.05$	$r=0.02$ $P>0.05$	I				
TGFβ1	$r=0.20$ $P>0.05$	$r=-0.28$ $P>0.05$	$r=0.09$ $P>0.05$	I			
TNFα	$r=0.01$ $P>0.05$	$r=0.03$ $P>0.05$	$r=0.18$ $P>0.05$	$r=-0.27$ $P>0.05$	I		
PDGF-ββ	$r=-0.08$ $P>0.05$	$r=-0.02$ $P>0.05$	$r=0.17$ $P>0.05$	$r=0.30$ $P>0.05$	$r=-0.21$ $P>0.05$	I	
CRP	$r=-0.16$ $P>0.05$	$r=0.12$ $P>0.05$	$r=0.04$ $P>0.05$	$r=-0.17$ $P>0.05$	$r=0.41$ $P=0.004^*$	$r=-0.16$ $P>0.05$	I
Epi	I			HAPE-c			
Norepi	$r=0.50$ $P=0.004^*$	I					
TH	$r=-0.16$ $P>0.05$	$r=-0.26$ $P>0.05$	I				
TGFβ1	$r=-0.14$ $P>0.05$	$r=-0.14$ $P>0.05$	$r=-0.08$ $P>0.05$	I			
TNFα	$r=-0.44$ $P=0.02$	$r=-0.31$ $P>0.05$	$r=0.45$ $P=0.01$	$r=-0.21$ $P>0.05$	I		
PDGF-ββ	$r=-0.22$ $P>0.05$	$r=-0.34$ $P>0.05$	$r=-0.04$ $P>0.05$	$r=0.40$ $P=0.03$	$r=-0.20$ $P>0.05$	I	
CRP	$r=0.46$ $P=0.01$	$r=-0.11$ $P>0.05$	$r=-0.02$ $P>0.05$	$r=-0.03$ $P>0.05$	$r=-0.31$ $P>0.05$	$r=0.10$ $P>0.05$	I
Epi	I			HLs			
Norepi	$r=0.85$ $P=9.78E-07^*$	I					
TH	$r=0.07$ $P>0.05$	$r=0.04$ $P>0.05$	I				
TGFβ1	$r=-0.14$ $P>0.05$	$r=-0.04$ $P>0.05$	$r=-0.02$ $P>0.05$	I			
TNFα	$r=0.24$ $P>0.05$	$r=0.03$ $P>0.05$	$r=0.48$ $P=0.03$	$r=-0.40$ $P>0.05$	I		
PDGF-ββ	$r=-0.54$ $P=0.01$	$r=-0.54$ $P=0.01$	$r=0.24$ $P>0.05$	$r=-0.08$ $P>0.05$	$r=0.15$ $P>0.05$	I	
CRP	$r=0.40$ $P>0.05$	$r=0.53$ $P=0.01$	$r=-0.21$ $P>0.05$	$r=-0.39$ $P>0.05$	$r=0.22$ $P>0.05$	$r=-0.20$ $P>0.05$	I

**Notes:** Sample size for biomarker correlations: HAPE-p, n=50; HAPE-c, n=33; HLs, n=23. \* $P<0.006$ , cutoff for significance.

**Abbreviations:** Epi, epinephrine; Norepi, norepinephrine; TH, tyrosine hydroxylase; TGFβ1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; PDGF-ββ, platelet-derived growth factor beta beta; CRP, C-reactive protein; HAPE, high-altitude pulmonary edema; HAPE-p, HAPE-patients; HAPE-c, HAPE-controls; HLs, highlanders.

## Results

### Clinical characteristics

The clinical characteristics of the three study groups are summarized in Table 3. Arterial oxygen saturation ( $\text{SaO}_2$ , 69.95%) was significantly lower ( $P<0.0001$ ) in HAPE-p compared to the levels in the other two healthy control groups, that is, HAPE-c (88.84%) and HLs (86.46%). Furthermore, HLs had comparatively lower  $\text{SaO}_2$  levels than HAPE-c. MAP was similar between HAPE-p and HAPE-c ( $P=0.41$ ), but it was elevated in HAPE-p and HAPE-c when

compared to HLs, respectively ( $P<0.0001$ ). BMI was significantly higher in HAPE-p compared to the two healthy control groups, HAPE-c and HLs ( $P\leq 0.01$ ).

### Biomarker evaluation

Blood plasma levels of epinephrine, norepinephrine, TH, TGFβ1, and TNFα were observed to be significantly increased in HAPE-p when compared with both the healthy groups, that is, HAPE-c and HLs ( $P<0.0001$ ; Table 1). Blood plasma levels of CRP were significantly decreased in

**Table 3** Clinical characteristics of the three study groups

Clinical characteristics	HAPE-c (n=203)	HAPE-p (n=204)	HLs (n=116)	P-value		
				HAPE-p versus HAPE-c	HAPE-p versus HLs	HLs versus HAPE-c
Age (years)	28.15±7.91	31.53±7.85	37.06±9.34	<0.0001	<0.0001	<0.0001
SaO <sub>2</sub> (%)	88.84±8.65	69.95±7.85	86.46±6.26	<0.0001	<0.0001	0.01
BMI (kg/m <sup>2</sup> )	20.34±3.39	25.32±4.63	21.23±2.42	<0.0001	<0.0001	0.01
MAP (mmHg)	94.87±10.88	95.64±7.81	89.61±10.66	0.41	<0.0001	<0.0001

**Notes:** The data are presented as mean ± SD. A one-way analysis of variance was used to calculate the P-values using the Epi Info™ software Version 6.

**Abbreviations:** HAPE, high-altitude pulmonary edema; HAPE-c, HAPE controls; HAPE-p, HAPE patients; HLs, highlanders; SaO<sub>2</sub>, arterial oxygen saturation; BMI, body mass index; MAP, mean arterial pressure; SD, standard deviation.

HAPE-p against HAPE-c ( $P=7.24E-14$ ; Table 1) and HLs ( $1.13E-22$ ; Table 1). The PDGF- $\beta\beta$  levels did not differ in HAPE-p when compared with the two healthy groups, that is, HAPE-c and HLs ( $P>0.01$ ; Table 1). In HLs, there was no difference in the levels of epinephrine, norepinephrine, and TH against HAPE-c ( $P>0.05$ ), while there was a marginally significant difference in the levels of TGF $\beta$ 1 ( $P=0.0002$ ), TNF $\alpha$  ( $P=0.04$ ), PDGF- $\beta\beta$  ( $P=0.01$ ), and CRP ( $P=0.004$ ) against HAPE-c (Table 1).

### Inter-biomarker correlations

In HAPE-p, a negative correlation between epinephrine and norepinephrine ( $r=-0.61$ ,  $P=4.6E-06$ ; Table 2) was observed, while a positive correlation was observed between epinephrine and norepinephrine in HAPE-c ( $r=0.50$ ,  $P=0.004$ ; Table 2) and HLs ( $r=0.85$ ,  $P=9.78E-07$ ; Table 2). A positive correlation was also observed between TNF $\alpha$  and CRP ( $r=0.41$ ,  $P=0.004$ ; Table 2) in HAPE-p, but the same was not significant in any of the other healthy groups, HAPE-c ( $r=-0.31$ ,  $P>0.05$ ; Table 2) and HLs ( $r=0.22$ ,  $P>0.05$ ; Table 2).

### Correlations between arterial oxygen saturation (SaO<sub>2</sub>) and biomarkers

In HAPE-p, no correlation was observed between the studied biomarkers and SaO<sub>2</sub> (Figure 2). In HAPE-c, a negative correlation was observed between SaO<sub>2</sub> and norepinephrine ( $r=-0.18$ ,  $P=0.01$ ; Figure 2B) and SaO<sub>2</sub> and TNF $\alpha$  ( $r=-0.41$ ,  $P=0.005$ ; Figure 2E). In contrast, a positive correlation was observed between SaO<sub>2</sub> and CRP ( $r=0.34$ ,  $P=0.02$ ; Figure 2G). In HLs, a positive correlation was observed between SaO<sub>2</sub> and norepinephrine ( $r=0.20$ ,  $P=0.04$ ; Figure 2B).

### Correlations between BMI and biomarkers

In HAPE-p, a negative correlation was observed between epinephrine and BMI ( $r=-0.24$ ,  $P=0.001$ ; Figure 3A), while a positive correlation was observed between norepinephrine and BMI ( $r=0.16$ ,  $P=0.03$ ; Figure 3B). In HAPE-c, a positive

correlation was observed between BMI and norepinephrine ( $r=0.22$ ,  $P=0.002$ ; Figure 3B) and BMI and TH ( $r=0.34$ ,  $P=0.02$ ; Figure 3C). In HLs, no correlation was observed between the studied biomarkers and BMI (Figure 3).

### Correlations between MAP and biomarkers

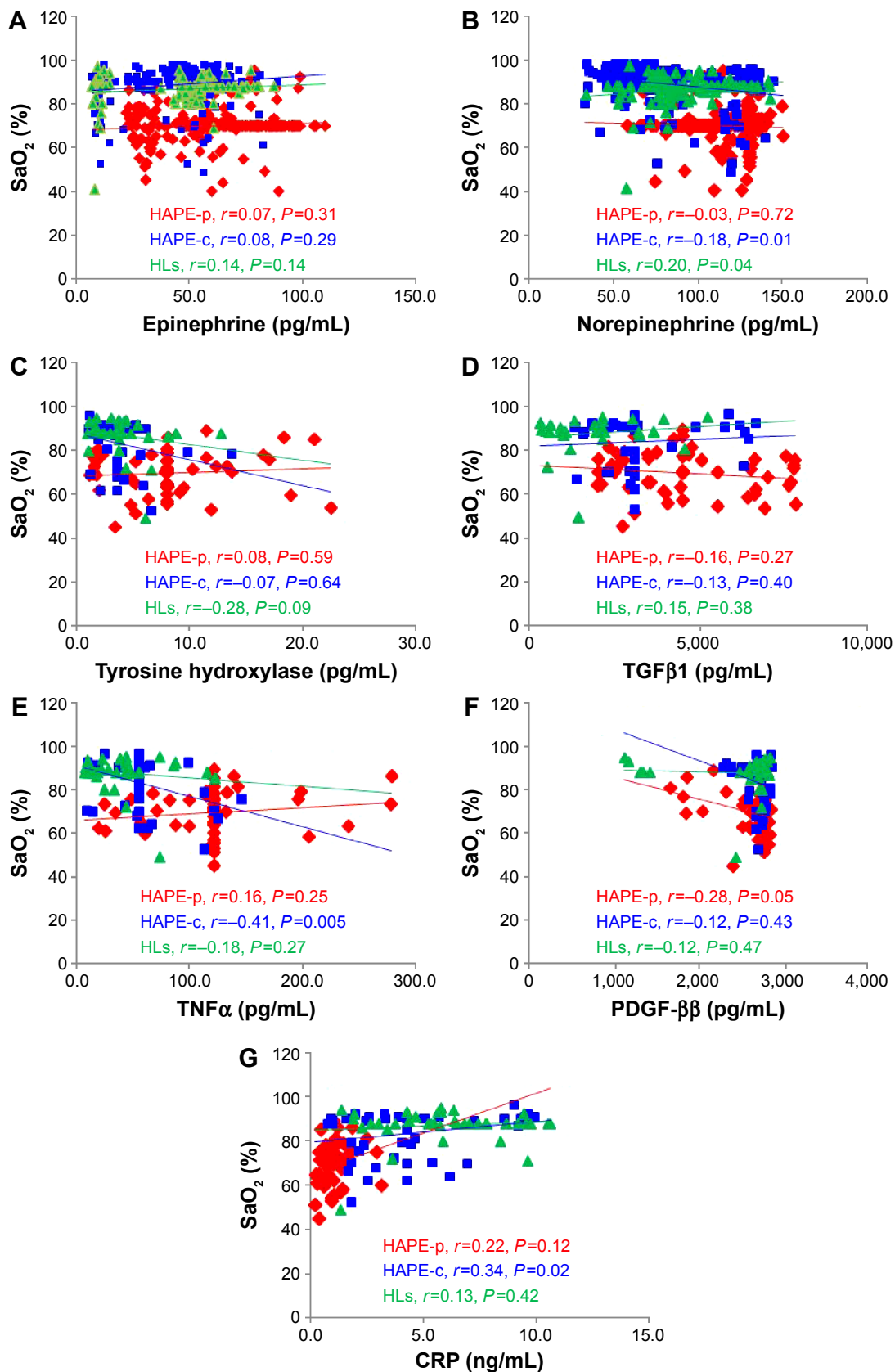
In HAPE-p, a significant positive correlation was observed between TNF $\alpha$  and MAP ( $r=0.33$ ,  $P=0.02$ ; Figure 4E). In HAPE-c, no correlation was observed between the studied biomarkers and MAP (Figure 4). In HLs, a significant positive correlation was observed between norepinephrine and MAP ( $r=0.20$ ,  $P=0.03$ ; Figure 4B).

### Predictive value of biomarkers

ROC curve analyses using binary logistic regression model yielded epinephrine, norepinephrine, TH, TGF $\beta$ 1, TNF $\alpha$ , and CRP as predictive/potential biomarkers for HAPE. In HAPE-p versus HAPE-c, AUC for the studied biomarkers was as follows: epinephrine (0.70, 95% CI =0.65–0.75,  $P=3.9E-12$ ), norepinephrine (0.76, 95% CI =0.72–0.81,  $P=3.0E-20$ ), TH (0.79, 95% CI =0.70–0.89,  $P=3.1E-07$ ), TGF $\beta$ 1 (0.71, 95% CI =0.61–0.81,  $P=0.0003$ ), TNF $\alpha$  (0.88, 95% CI =0.80–0.95,  $P=4.3E-12$ ), and CRP (0.94, 95% CI =0.89–0.98,  $P=3.2E-14$ ) (Figure 5). In HAPE-p versus HLs, AUC for the studied biomarkers was as follows: epinephrine (0.71, 95% CI =0.65–0.76,  $P=1.2E-09$ ), norepinephrine (0.75, 95% CI =0.69–0.81,  $P=1.0E-13$ ), TH (0.77, 95% CI =0.66–0.87,  $P=0.00001$ ), TGF $\beta$ 1 (0.88, 95% CI =0.82–0.95,  $P=2.7E-10$ ), TNF $\alpha$  (0.91, 95% CI =0.85–0.98,  $P=1.3E-11$ ), and CRP (0.99, 95% CI =0.97–1.00,  $P=9.0E-16$ ) (Figure 6). The AUC for PDGF- $\beta\beta$  was <0.70 in both HAPE-p versus HAPE-c and HAPE-p versus HLs.

### Discussion

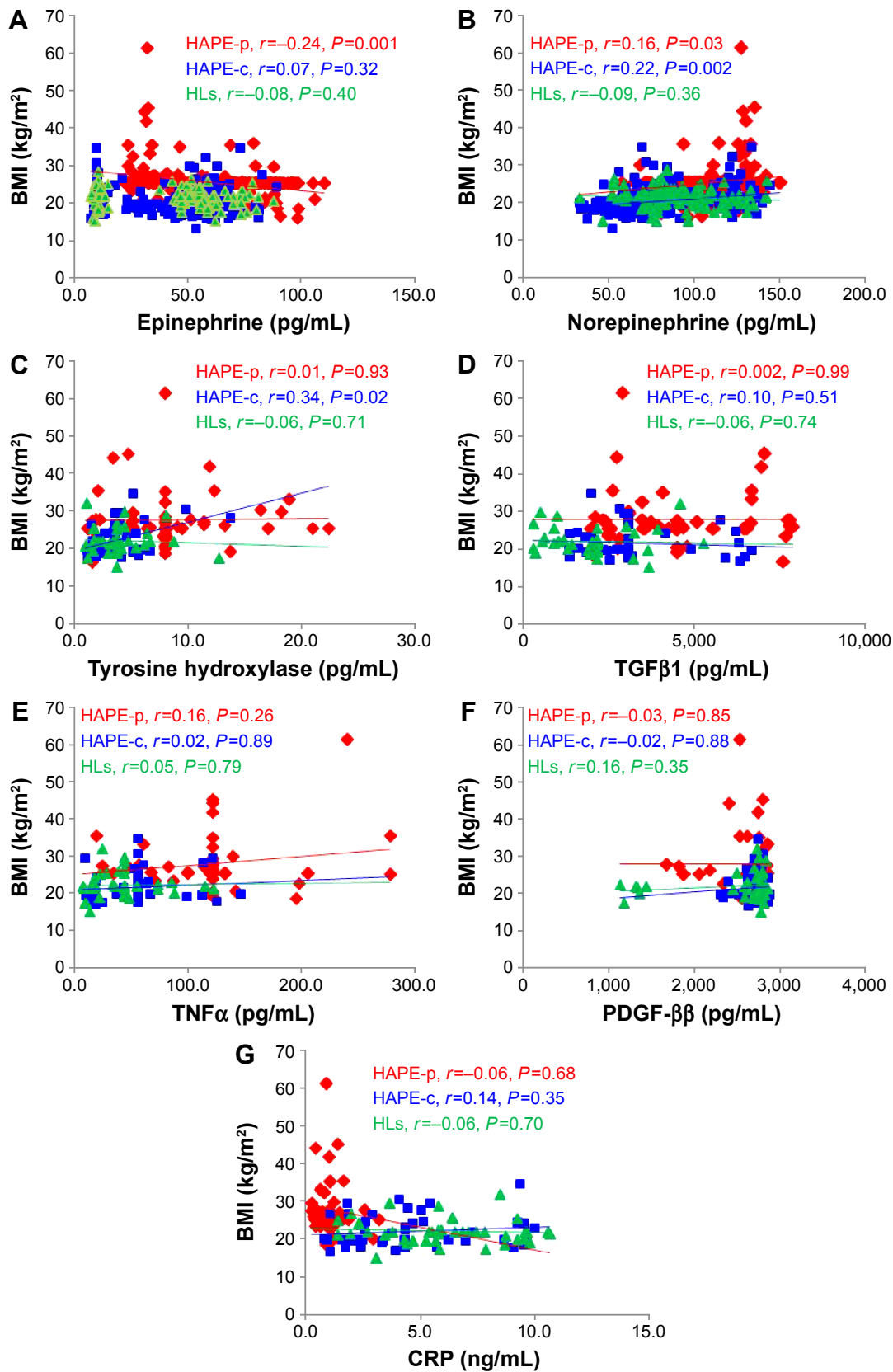
A biomarker is a measurable indicator of any biological state or condition. This study has provided an understanding of



**Figure 2** Correlation analysis between SaO<sub>2</sub> and biomarkers in the three study groups.  $P < 0.05$  was considered significant.

**Notes:** Sample size for biomarker correlations: HAPE-p,  $n=50$ ; HAPE-c,  $n=33$ ; HLs,  $n=23$ . Epinephrine (A), norepinephrine (B), tyrosine hydroxylase (C), TGF $\beta$ 1 (D), TNF $\alpha$  (E), PDGF- $\beta\beta$  (F), and CRP (G).

**Abbreviations:** SaO<sub>2</sub>, arterial oxygen saturation; HAPE, high-altitude pulmonary edema; HAPE-p, HAPE-patients; HAPE-c, HAPE-controls; HLs, highlanders; TGF $\beta$ 1, transforming growth factor beta 1; TNF $\alpha$ , tumor necrosis factor alpha; PDGF- $\beta\beta$ , platelet-derived growth factor beta beta; CRP, C-reactive protein.

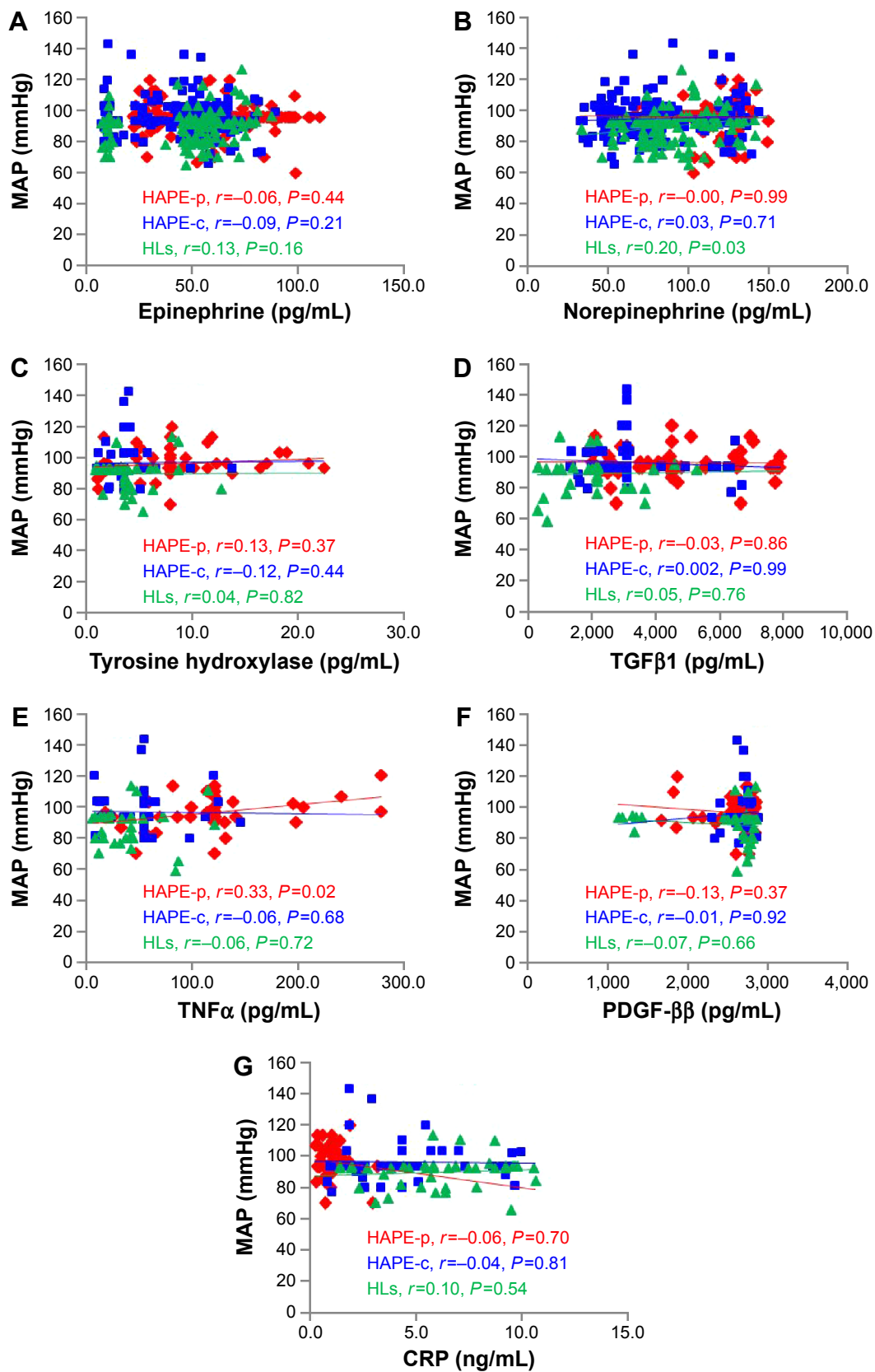


**Figure 3** Correlation analysis between BMI and biomarkers in the three study groups.

**Notes:** Sample size for biomarker correlations: HAPE-p, n=50; HAPE-c, n=33; HLLs, n=23. Epinephrine (A), norepinephrine (B), tyrosine hydroxylase (C), TGFβ1 (D), TNFα (E), PDGF-ββ (F), and CRP (G).

**Abbreviations:** BMI, body mass index; HAPE, high-altitude pulmonary edema; HAPE-p, HAPE-patients; HAPE-c, HAPE-controls; HLLs, highlanders; TGFβ1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; PDGF-ββ, platelet-derived growth factor beta beta; CRP, C-reactive protein.

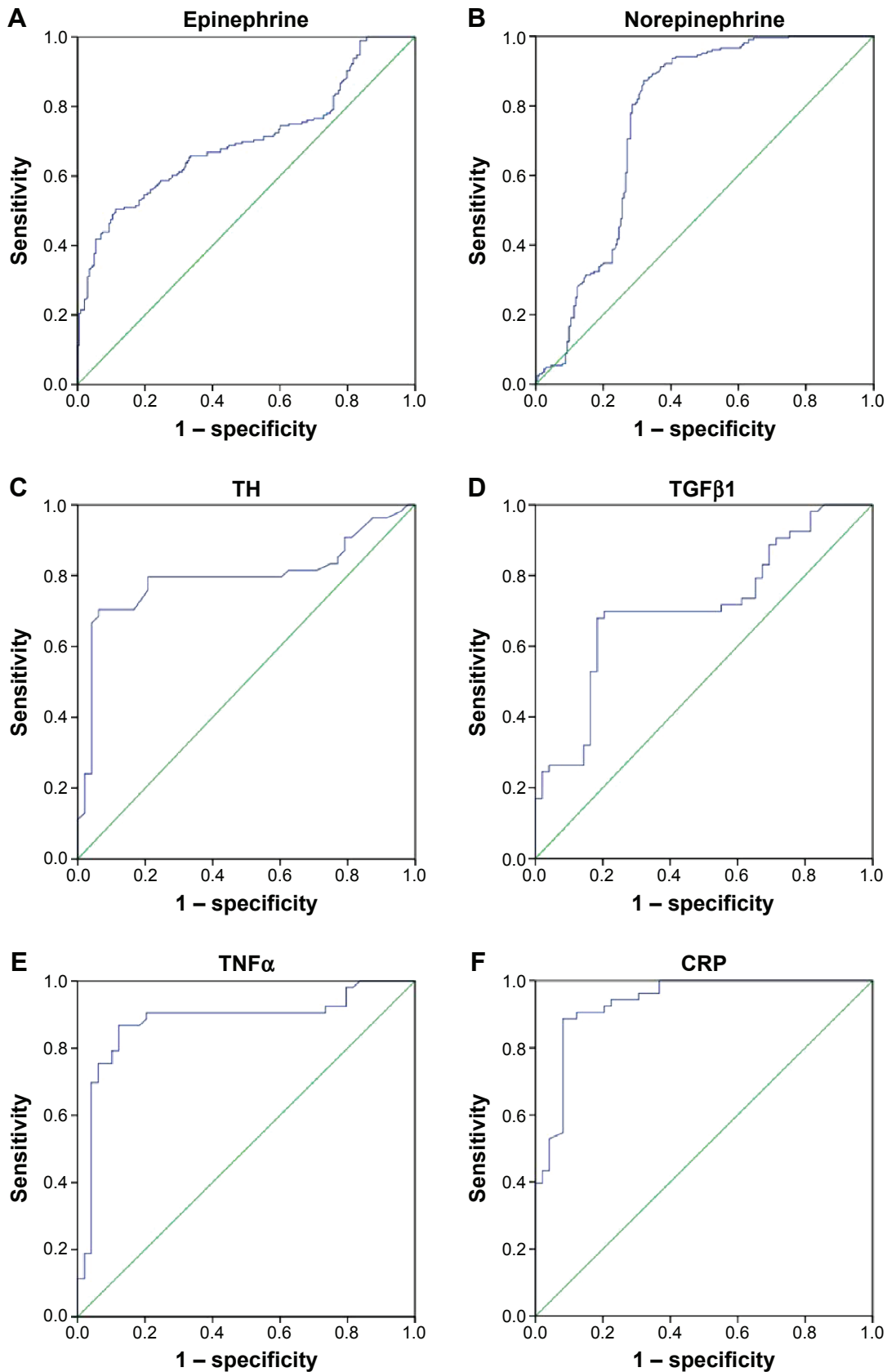




**Figure 4** Correlation analysis between MAP and biomarkers in the three study groups.

**Notes:** Sample size for biomarker correlations: HAPE-p,  $n=50$ ; HAPE-c,  $n=33$ ; HLs,  $n=23$ . Epinephrine (A), norepinephrine (B), tyrosine hydroxylase (C), TGF $\beta$ 1 (D), TNF $\alpha$  (E), PDGF- $\beta\beta$  (F), and CRP (G).

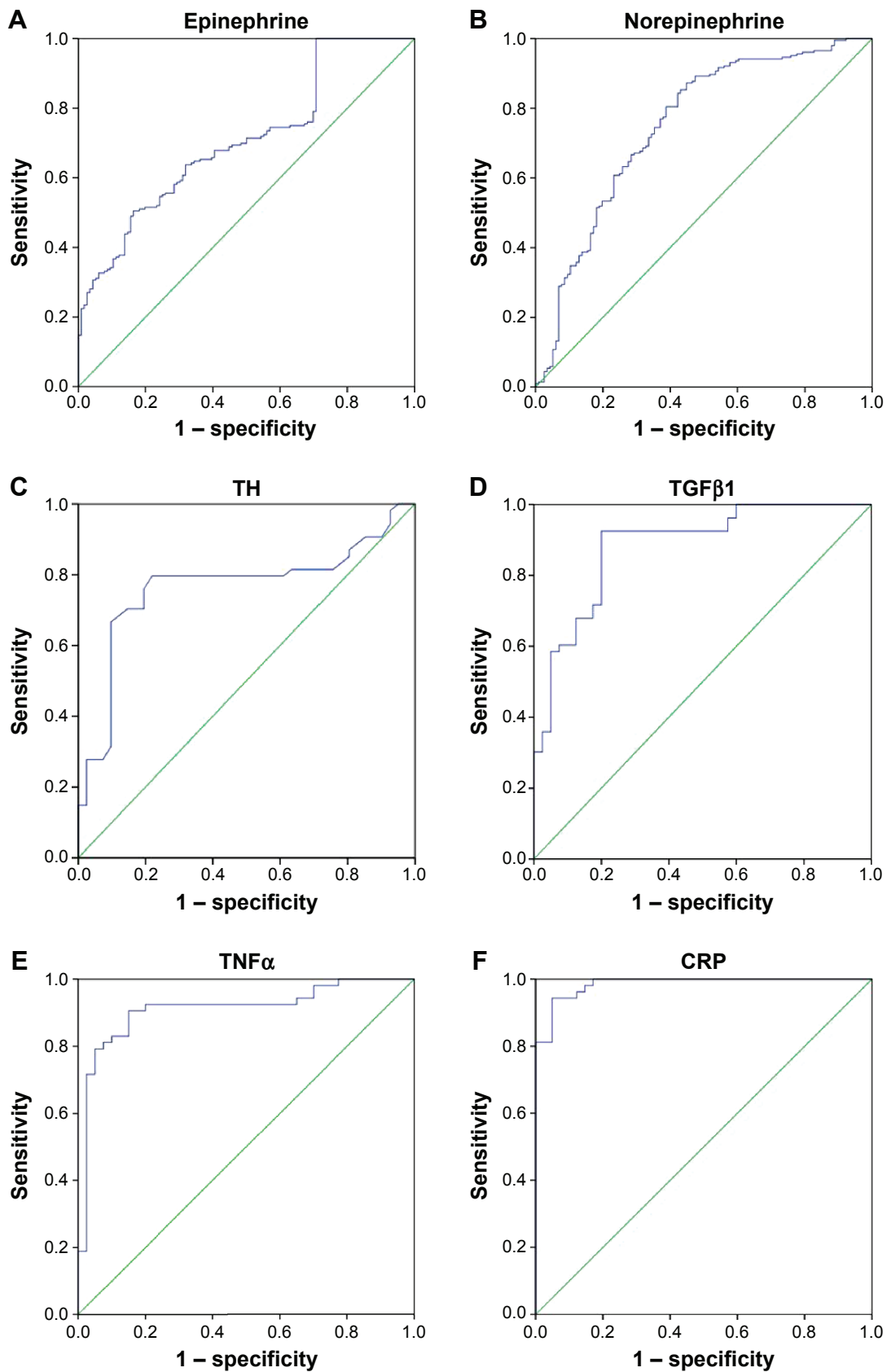
**Abbreviations:** MAP, mean arterial pressure; HAPE, high-altitude pulmonary edema; HAPE-p, HAPE-patients; HAPE-c, HAPE-controls; HLs, highlanders; TGF $\beta$ 1, transforming growth factor beta 1; TNF $\alpha$ , tumor necrosis factor alpha; PDGF- $\beta\beta$ , platelet-derived growth factor beta beta; CRP, C-reactive protein.



**Figure 5** ROC curves for determining the prediction value of the studied biomarkers.

**Notes:** AUC >0.70 was considered as benchmark for good predictive value of the biomarker. The analysis has been performed between HAPE-p and HAPE-c. Epinephrine (A), norepinephrine (B), TH (C), TGFβ1 (D), TNFα (E), and CRP (F).

**Abbreviations:** ROC, receiver operating characteristic; AUC, area under the curve; HAPE, high-altitude pulmonary edema; HAPE-p, HAPE-patients; HAPE-c, HAPE-controls; TH, tyrosine hydroxylase; TGFβ1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; CRP, C-reactive protein.



**Figure 6** ROC curves for determining the prediction value of the studied biomarkers.

**Notes:** AUC >0.70 was considered as benchmark for good predictive value of the biomarker. The analysis has been performed between HAPE-p and HLs. Epinephrine (A), norepinephrine (B), TH (C), TGFβ1 (D), TNFα (E), and CRP (F).

**Abbreviations:** ROC, receiver operating characteristic; AUC, area under the curve; HAPE, high-altitude pulmonary edema; HAPE-p, HAPE-patients; HLs, highlanders; TH, tyrosine hydroxylase; TGFβ1, transforming growth factor beta 1; TNFα, tumor necrosis factor alpha; CRP, C-reactive protein.

the biochemical parameters under the stressful environment of hypobaric hypoxia at HA in HAPE subjects. To the best of our knowledge, this study is first of its kind with these molecules being studied in the three study groups. We do admit that more functional studies need to be done with higher number of samples to achieve more meaningful findings. But ours is a preliminary study, and we have made an effort to provide a glimpse of altered biochemical parameters in HAPE and tried to deduce the same and associate with biological findings.

Evaluation of clinical characteristics among the three groups revealed decreased  $\text{SaO}_2$  ( $<80\%$ ) and increased MAP ( $>95$  mmHg) in HAPE-p. In contrast, the two healthy control groups, HAPE-c and HLs, had  $\text{SaO}_2$  ( $>85\%$ ) and MAP ( $<95$  mmHg). Decrease in  $\text{SaO}_2$   $<80\%$  leads to hypoxemia that in turn impairs normal functioning of vital organs such as lungs, heart, brain, and kidneys.<sup>31</sup> Moreover, MAP is a measure of systemic vascular resistance.<sup>32</sup> The findings suggest that hypoxemia and increased vascular resistance in HAPE-p lead to lung dysfunction and exaggerated vasoconstriction, a hallmark of HAPE.<sup>1-3</sup> The lower  $\text{SaO}_2$  in HLs (86.46%) against lowland control HAPE-c (88.84%) suggested that the native population was under stress. However, despite this stress, HLs do not suffer from any disorder, indicating that the population is adapted to the environment of HA; the results are in concordance with the earlier findings on adaptation in HLs.<sup>33,34</sup>

Adrenomedullary hormones, epinephrine and norepinephrine, are the products of activated sympathetic nervous system,<sup>35</sup> and TH is a rate-limiting enzyme in the synthesis of epinephrine and norepinephrine.<sup>36</sup> Significantly elevated levels of epinephrine and norepinephrine and TH in HAPE-p specified an increase in sympathetic nervous system activity in HAPE. Increased TH levels in HAPE-p also suggested a higher formation of these catecholamines in HAPE. The levels of epinephrine, norepinephrine, and TH in the HLs were less than those in the HAPE-p, suggesting that an increase in sympathetic nervous system activity is critical for determining an individual's predisposition to HAPE. Epinephrine induces pulmonary edema;<sup>37,38</sup> its elevated levels in HAPE again confirm this fact.

TGF $\beta$ 1, a regulator of collagen deposition and other extracellular matrix proteins, such as fibronectin and plasminogen activator inhibitor 1, in vascular smooth muscle and endothelial cells lining the blood vessel, was observed to be increased in HAPE-p against the two healthy control groups.<sup>20-22,39</sup> Increased deposition of collagen and other extracellular matrix proteins leads to the increased airway fibrosis

or vascular remodeling, further decreasing the pulmonary capacity, a hallmark of HAPE.<sup>1-3,20-22,40</sup> In addition, TNF $\alpha$  is a pleiotropic proinflammatory cytokine that is known to induce vascular smooth muscle cell proliferation and reactive oxygen species production leading to oxidative stress, a feature commonly associated with HAPE.<sup>26-28</sup> Less or nonsignificant *P*-values between HAPE-c and HLs demonstrated that even though HLs and HAPE-c had small differences in the levels of these circulatory biomarkers, these levels were too close to protect against HAPE. In this study, stress-activated inflammatory marker, CRP, was observed to be protective against HAPE as the CRP levels were significantly reduced in HAPE-p against the two healthy groups, HAPE-c and HLs. The results were in sharp contrast to the initial hypothesis and earlier studies related to the CRP levels under HA exposure.<sup>41,42</sup> More studies are needed in this direction with verification in larger sample size.

Correlation analysis is an important tool to understand the behavior of biomarkers with respect to one another. Thus, in this study, inter-biomarker correlations were performed along with clinical parameter–biomarker correlation to elucidate the effect of biomarkers on each other and also of clinical parameters such as  $\text{SaO}_2$  (arterial oxygen saturation), BMI, and MAP on biomarkers or vice versa. One of the limitations of our study has been less number of overlapping samples available for inter-biomarker correlation studies, but we had kept rigorous statistical cutoffs to deduce the current findings.

A negative correlation between epinephrine and norepinephrine in HAPE-p is evident from the fact that norepinephrine is a major binder for alpha 1-adrenergic receptors, so much of it is binding to the receptors and thus less is available in case of HAPE-p to be converted to epinephrine.<sup>35</sup> Whereas in HAPE-c and HLs, the correlation was positive, this could be due to the normal functioning of the pathway (norepinephrine getting converted to epinephrine smoothly) in the two healthy control groups. In addition, a positive correlation between TNF $\alpha$  and CRP in HAPE-p could be due to the fact that CRP formation is enhanced in the presence of TNF $\alpha$ .<sup>43</sup> With our results, we were not able to deduce much about the phenomenon of inflammation in HAPE. As both anti-inflammatory TGF $\beta$ 1 and proinflammatory TNF $\alpha$  were observed to be increased in HAPE-p, the acute marker of inflammation, CRP, was observed to be decreased.

A normal response of body to hypobaric hypoxic conditions of HA is hypoxic pulmonary vasoconstriction that increases the amount of high oxygen blood redirected to lungs. In HLs, a significant positive correlation between

SaO<sub>2</sub> and norepinephrine was observed, indicating that increased norepinephrine in the permanent residents might be involved in increasing arterial oxygen saturation. But an inverse correlation between norepinephrine and SaO<sub>2</sub>, TNF $\alpha$  and SaO<sub>2</sub>, and PDGF- $\beta\beta$  and SaO<sub>2</sub> in lowlanders, that is, HAPE-c and HAPE-p, signified that these stress-activated moieties are involved in causing exaggerated vasoconstriction that leads to decreased arterial oxygen saturation due to less amount of oxygen being allowed to stay in the lung vascular beds. CRP was observed to have a positive correlation with SaO<sub>2</sub>, again confirming its protective nature in HAPE.

Body fat is a major determinant of sympathetic nervous system activity.<sup>44</sup> A positive correlation between norepinephrine and TH with BMI in the lowlanders suggested that high body weight might be a factor associated with increased risk of HAPE. Epinephrine levels were observed to be negatively correlated with BMI indicating that fatty acid oxidation, that is, the process of energy metabolism might be altered in HAPE, leading to weight gain in HAPE-p. The results of this study were in concert with the previous findings by Sharabi et al.<sup>45</sup> In HLs, we did not observe any biomarker–BMI correlation, again suggesting the population being adapted to HA.

TNF $\alpha$  causes vasoconstriction by decreasing the generation of critical vasodilator nitric oxide<sup>46</sup> and increasing the secondary release of vasoconstrictor endothelin-1.<sup>47</sup> Decreased nitric oxide increases systemic vascular resistance,<sup>48,49</sup> and in our study, we have found MAP, a measure of systemic vascular resistance and cardiac output, to be positively correlating with TNF $\alpha$ . The finding indicates that the phenomenon of exaggerated vasoconstriction is prevalent in HAPE-p, and TNF $\alpha$  might be one of the markers associated with the process.

ROC curve analysis is an important graphical measure to assess sensitivity and specificity of a prediction deduced via a statistical model.<sup>50</sup> ROC curve analysis on the seven studied biomarkers yielded six biomarkers, namely, epinephrine, norepinephrine, TH, TGF $\beta$ 1, TNF $\alpha$ , and CRP, showing good prediction value for HAPE with AUC >0.70. This finding supported our results that these biomarkers are associated with HAPE and could prove as potential predictive biomarkers. Nonetheless, we admit that verification in larger sample size is needed.

## Conclusion

Among the seven studied biomarkers, six biomarkers, epinephrine, norepinephrine, TH, TGF $\beta$ 1, TNF $\alpha$ , and CRP, could serve as plausible candidates for therapeutic

interventions in HAPE. Nevertheless, we admit that more studies are needed in this field pertaining to these parameters with larger sample sizes.

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## Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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