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Changes in Adenosine Metabolism in Asthma. A Study on Adenosine, 5′-NT, Adenosine Deaminase and Its Isoenzyme Levels in Serum, Lymphocytes and Erythrocytes

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Abstract

Background: Adenosine deaminase (ADA) and 5′-nucleotidase (5′-NT) play a crucial role in adenosine metabolism in healthy individuals. Adenosine is an inflammatory mediator of asthma. Changes in adenosine metabolism and role of ADA and 5′-NT in regulating adenosine level in asthmatics and correlation of these changes with severity of asthma are not clearly understood. Methods: In this study, we screened 5217 patients, of which 2416 were diagnosed with asthma. Further, of 2416 asthmatics, only 45 patients who strictly fulfilled the selection criteria were enrolled in the study. The patients were classified into mild, moderate and severe persistent groups; each group consisted of fifteen patients. Fifteen healthy subjects served as controls. Adenosine levels and activities of 5′-NT, total ADA, ADA1 and ADA2 in serum, lymphocytes and erythrocytes were determined. The data were analysed statistically and $p < 0.05$ was considered significant. Results: In asthma, adenosine levels in serum, lymphocytes and erythrocytes were found to be raised significantly. A significant reciprocal correlation existed between adenosine levels in serum, lymphocytes and erythrocytes of asthmatics and FEV1%. The 5′-nucleotidase activity in serum and lymphocytes was raised in moderate and severe persistent groups and an inverse correlation existed between 5′-nucleotidase activity and FEV1% whereas in erythrocytes it was raised only in severe persistent group and FEV1% had no correlation with the 5′-nucleotidase activity. The activities of total ADA, ADA1 and ADA2 were decreased in serum and lymphocytes of moderate and severe persistent asthmatics and a positive correlation existed between total ADA and FEV1%. In eryth-

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rocytes, total ADA activity increased in mild persistent group but remained unchanged in moderate and severe persistent groups. Conclusion: The present study suggests that adenosine levels tend to increase in serum, lymphocytes and erythrocytes with the severity of bronchial asthma. The balance between ADA and 5′-NT determines the levels of adenosine in serum and lymphocytes which may result in pathogenesis of asthma, or *vice versa*.

Keywords

Asthma, Adenosine, Metabolism, Adenosine Deaminase, 5′-Nucleotidase

1. Introduction

Bronchial asthma is one of the most common chronic diseases globally and currently affects nearly 300 million people [1]. Prevalence of asthma in Indian population is 2.38% as per a study conducted by Jindal in 2007 [2]. Airway inflammation plays a cardinal role in pathogenesis of bronchial asthma and airway hyperresponsiveness is the characteristic physiologic abnormality that determines excessive bronchoconstrictor response to multiple inhaled triggers [3]. There is good evidence that the specific pattern of airway inflammation in asthma is associated with airway hyperresposiveness that is further correlated with variable airflow obstruction [4]. Several cell types such as lymphocytes, mast cells, macrophages, eosinophils, neutrophils and epithelial cells produce inflammatory changes by release of various mediators like adenosine, histamine, kinin, leukotrienes, prostaglandins, PAF, chemokines and cytokines which interact in a complex way to produce airway inflammation [4]. Severity of asthma is based on the frequency and intensity of symptoms. The mildest grade is characterized by acute attacks upon allergen exposure, and with symptoms reversed by β-adrenergic agonists [5]; lung function is normal between acute attacks. More severe grades of asthma are characterized by sustained increase in airway resistance (the late-phase response), impaired basal lung function and heightened airways responsiveness to nonspecific irritants and cellular inflammation comprising of increased number of mast cells and eosinophils in the bronchial mucosa [6].

Adenosine is a ubiquitous purine nucleoside, playing a fundamental role in many biological processes such as energy generation and protein metabolism, but in the last two decades, it has become clear that adenosine is a mediator involved in the pathogenesis of many inflammatory disorders including bronchial asthma. It has been known for a long time that inflammatory tissue damage is accompanied by accumulation of extracellular adenosine in inflamed areas due to its release from non-immune and immune cells; local tissue hypoxia in inflamed areas represents one of the most important conditions leading to adenosine release and accumulation [7] [8]. Also, contributing to the accumulation of adenosine is the release of rapidly metabolized ADP and ATP from various cells including platelets, mast cells, and endothelial cells [9]. Adenosine, thus accumulated then interacts with specific G-protein coupled adenosine receptors (ARs) viz. A_1R, $A_{2A}R$, $A_{2B}R$, A_3R on inflammatory and immune cells to regulate their functions [10].

Evidence for role of adenosine in bronchial asthma was first demonstrated more than twenty years ago when a group of asthma patients exhibited bronchoconstriction in response to aerosolized adenosine while normal individuals didn't display response [11]. Adenosine acts through its A_{2B} and A_3 receptors to cause degranulation of mast cells and the release of vasoactive, pro-inflammatory and nociceptive mediators that include histamine, cytokines and proteolytic enzymes [12]-[15]. These mediators can cause bronchoconstriction in asthmatics. In addition to increase in mast cells activation, adenosine promotes release of inflammatory cytokines from smooth muscles, leukocytes chemotaxis via adenosine A2B receptors and eosinophils recruitment and activation via adenosine A3 receptors [16]. Adenosine seems to play an important role in modulation of T lymphocyte functions. Both peripheral cytotoxicT lymphocytes (CTLs) and T helper (Th) cells express A2A, A2B and A3 receptors, but A2A receptors are proposed to be the predominantly expressed in peripheral T lymphocytes [17], probably, extracellular adenosine effect on these cells is mediated through A2A receptors [18]. Moreover, cytokine production by activated Th cells is modulated by extracellular adenosine through A2A receptors [17]. Patients with bronchial asthma also reveal appreciable alterations of erythrocyte morphology and decreased membrane microviscosity which is related with severity of bronchial asthma [19] [20]. Adenosine levels are increased in the bronchoalveolar lavage (BAL) fluid of asthmatics compared to normal subjects, suggesting that there is an

imbalance in the homeostatic mechanisms involved in purine metabolism [21]. Furthermore, adenosine levels are increased in the lung lavage fluid after specific allergen challenge in sensitised rabbits [22]. Inhalation of allergen in atopic asthmatics increases adenosine plasma levels up to 3-fold [23]. Elevated concentrations of adenosine in the lungs of adenosine deaminase deficient mouse models have produced a lung phenotype with features of asthma [24]. The specific cellular source of adenosine in bronchoalveolar lavage fluid is unknown, but is likely to include mast cells along with epithelial cells, neutrophils, lymphocytes and platelets [25].

Adenosine bioavailability is a key determinant of its action. The processes related to its production, release, cellular uptake and metabolism determine the bioavailability of adenosine at receptor sites [26]. Adenosine occupies an interesting central position in purine metabolism. It may be subjected to three metabolic fates: phosphorylation to the nucleotide level, deamination to inosine, or conversion to the base level by adenosine phosphorylase. But adenosine phosphorylase has been found only in bacterial systems [27] [28]. Phosphorylation is favoured at low concentrations while deamination predominates at higher adenosine concentrations [29]. Under normal conditions adenosine is derived from intracellular adenosine monophosphate (AMP) that is present at low levels in the cell, mostly derived from the catabolism of high energy adenosine phosphates (ATP, ADP) [30]. Intracellular (AMP) is formed and shortly reconverted to ADP and ATP as part of the energy cycle. However, under conditions of high-energy demand, AMP cannot be reconverted and it is metabolised to adenosine by 5′-nucleotidase (plasma membrane bound mainly as well as cytoplasmic) [31]. Intracellular levels of adenosine are kept low by its conversion to AMP by the enzyme adenosine kinase and to inosine by adenosine deaminase, but when energy demands are greater as in inflammation, deamination predominates [32]. Extracellular adenosine diffuses back into the cell through the operation of an energy-independent nucleoside transporter [33]. 5′-nucleotidase (5′-NT, E.C. 3.1.3.5) catalyzes the hydrolysis of the phosphoric ester bond of 5′-ribonucleotides to the corresponding ribonucleoside and phosphate. The main function of 5′-NT is the hydrolysis of AMP to adenosine. Adenosine deaminase (ADA, EC 3.5.4.4), a key enzyme in the purine salvage pathway, catalyzes the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. Two different isoenzymes of ADA designated as ADA1 and ADA2 were found in mammals and lower vertebrates [34].

The production, release and metabolism of adenosine depend ultimately on the relevant enzyme activities influencing its metabolism. Adenosine deaminase and 5′-nucleotidase are the two key enzymes regulating adenosine level inside the cell as well as in plasma. We hypothesized that the decreased activity of adenosine deaminase or increased activity of 5′-nucleotidase or both may result in elevated level of adenosine. Since studies measuring activities of these enzymes and correlating it with adenosine levels in bronchial asthma patients are lacking, we proposed the present study to examine the activities of adenosine deaminase, its isoenzymes, 5′-nucleotidase and adenosine levels in serum, erythrocytes and lymphocytes to understand the metabolism of adenosine in bronchial asthma.

2. Materials and Methods

2.1. Plan of Study

The study included a total number of 60 subjects of bronchial asthma and healthy controls. The asthmatic patients were classified into three groups consisting of 15 patients each viz. mild persistent, moderate persistent and severe persistent as per the EPR 3 guidelines [35]. The control group consisted of 15 healthy subjects. Blood (10 ml) was collected, serum, lymphocytes and erythrocytes separated, and adenosine levels, assay of activities of 5′-NT, ADA, ADA1 and ADA2 were performed and correlated with FEV1 (% of predicted). The study was approved by the Ethics Committee of Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India. Informed and written consent was taken from each subject.

2.2. Study Population

The patients were selected from those attending the outpatient department of Vallabhbhai Patel Chest Institute. During the period of this study, 5217 patients attended outpatient department, out of which 2416 were diagnosed as patients of bronchial asthma. We selected 45 patients from 2416 asthmatics according to our inclusion and exclusion criteria. Inclusion criteria for asthmatics: Patients with reversible airflow obstruction and pulmonary function test as per the EPR 3 guidelines [35], with age between 18 and 60 years of either sex. Exclusion criteria: smokers, patients having other respiratory and systemic disease, on oral corticosteroids, and pregnant or lactating females. Fifteen age and sex matched healthy subjects were included in the study.

2.3. Chemicals and Reagents

Adenosine deaminase, EHNA [Erythro-9(2-hydroxy-3-nonyl) adenine], histopaque (specific gravity −1.077), and 4-methylaminophenol sulphate were purchased from Sigma Chemical Co. St Louis, USA. Adenosine, adenosine monophosphate, nickel chloride, potassium bisulphate, and sodium nitroprusside were procured from Sisco Research Laboratories PVT. LTD. (Mumbai, India). Ammonium molybdate, ammonium sulphate, disodium hydrogen phosphate, EDTA (Ethylenediaminetetraacetic acid) disodium, manganese sulphate, perchloric acid 70%, phenol, potassium carbonate, sodium acetate, sodium dihydrogen phosphate, sodium hydroxide, sodium hypochlorite, sodium veronal, sulphuric acid 96%, trichloroacetic acid and triethanolamine hydrochloride were purchased from Qualigens Fine Chemicals (Mumbai, India). All other chemicals used were of analytical grade.

2.4. Preparation of Serum and Lysate of Lymphocytes and Erythrocytes

Venous blood (10 ml) was collected under aseptic conditions in two tubes, 7 ml of it was collected in tube containing EDTA (1 mg/ml) as anticoagulant and remaining 3 ml was taken in other tube without anticoagulant to isolate the serum. Lymphocytes were isolated by the method of Boyum [36] with some modifications. Venous blood (7 ml) was diluted in the ratio of 1:1 with physiological saline (0.15 M NaCl), carefully layered over 7 ml histopaque (specific gravity 1.077) and centrifuged at 2000 rpm (670 × g) in a refrigerated centrifuge (Plasto Craft, 4 R-V/FM, India) at 4˚C using swing out rotor (no. 15) for 15 minutes. The opaque ring at the interface containing lymphocytes was collected with a sterile Pasteur pipette, diluted with physiological saline and centrifuged at 1500 rpm (500 × g) in a refrigerated centrifuge (Plasto Craft, 4 R-V/FM, India) at 4˚C for 10 min. The pellet containing lymphocytes was washed twice with physiological saline. Contaminating erythrocytes were removed by giving osmotic shock to the pellet by the method of Bansal *et al.* [37]. The cell suspension was centrifuged again, the pellet washed twice with physiological saline and resuspended in physiological saline. The lymphocytes were counted using automated cell counter (Sysmex PoCH-100i). After removal of opaque ring at the interface containing lymphocytes and discarding the supernatant, packed erythrocytes were washed with 5 volumes of physiological saline (0.15 M NaCl) and centrifuged at 3000 rpm (650 × g) in a refrigerated centrifuge (Plasto Craft, 4 R-V/FM, India) using angular rotor (no. 9) at 4˚C for 10 minutes. Pellet was washed three times similarly. Supernatant was discarded each time. The packed cells were resuspended in 0.15 M saline. The erythrocytes were counted using automated cell counter (Sysmex PoCH-100i). To assess the overall viability of lymphocytes, the cell suspension was washed two times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and treated with a 0.4% solution of trypan blue for 2 to 3 min. Lymphocytes and erythrocytes were suspended in phosphate buffer (50 mM NaH_2PO_4 and $Na_2HPO_4{\cdot}12H_2O$, pH 6.5) for adenosine deaminase and its isoenzymes assay, in veronal buffer (40 mM $C_8H_{11}O_3N_2Na$, pH 7.5) for 5′-nucleotidase assay and in triethanolamine buffer (0.1 M $C_6H_{15}NO_3$, pH 7.4) for adenosine estimation. Lymphocytes suspended in respective buffer to obtain a suspension of 20×10^6 cells/ml were sonicated by an ultrasonicator using a microtip by giving five bursts of 30 seconds each at an interval of one minute at 0˚C in an ice bath. The cell lysis was confirmed by light microscopy. Venous blood (3 ml) collected in tube without anticoagulant was allowed to clot and then centrifuged at 2700 rpm (900 × g) in a refrigerated centrifuge (Plasto Craft, 4 R-V/FM, India) using swing out rotor (no. 15) at 4˚C for 10 minutes to obtain serum.

2.5. Adenosine Estimation

Adenosine estimation was done according to the method described by Mollering and Bergmeyer [38]. Samples were deproteinized with 70% PCA. In a 3 ml eppendorf tube 600 µl of sample and 600 µl of ice cold 70% PCA (1 M) was mixed thoroughly and centrifuged at 3080 rpm (1000 × g) in a refrigerated centrifuge (Hermle Labortechnik GmbH Germany Type Z 36 HK) using angular rotor (no. 8) for 15 minutes at 10˚C. Then to 400 µl of supernatant, 20 µl K_2CO_3 solution (5 M) was added to neutralize the sample. The reaction was allowed to stand for 15 minutes in an ice bath, centrifuged at 3080 rpm (1000 × g) at 10˚C for 15 minutes and supernatant saved for adenosine estimation. Triethanolamine buffer solution (0.1 M $C_6H_{15}NO_3$, pH 7.4) (1000 µl) and sample (200 µl) was pipetted into cuvette, mixed thoroughly, and extinction E_I was read at 265 nm wavelength, using UV-Vis spectrophotometer (Cary Bio 100 Varian). Then, 8 µl of adenosine deaminase (0.1 mg protein/ml) was added and it was kept at 37˚C for 5 minutes in a water bath. Final extinction E_{II} was read after 5 minutes. The difference in extinction coefficient ($\Delta E = E_I - E_{II}$) was calculated. The extinction increase due to addition of

adenosine deaminase suspension alone was determined by the addition of further 8 µl of adenosine deaminase suspension II at the end of the reaction. Then this extinction increase was added to the extinction difference (ΔE). The results were expressed in nmol adenosine/ml serum, nmol adenosine/million lymphocytes and nmol adenosine/billion erythrocytes.

2.6. 5′-Nucleotidase (5′-NT) Assay

5′-nucleotidase activity was determined by the method described by Gerlach and Hiby [39]. Five tubes were taken and labelled, I as blank, II as standard, III without Ni^{2+}, IV with Ni^{2+} and V as control, followed by pipetting in eppendorf tubes (3 ml): distilled water (800 µl) in I and II, veronal buffer (40 mM $C_8H_{11}O_3N_2Na$, pH 7.5) (520 µl in serum and 640 µl in lysates of lymphocytes and erythrocytes labelled tubes) in III and V tubes and veronal buffer (40 mM $C_8H_{11}O_3N_2Na$, pH 7.5) (440 µl in serum and 560 µl in lysates of lymphocytes and erythrocytes labelled tubes) in IV tube, manganese sulphate solution (20 mM) (40 µl) in III, IV and V tubes, nickel chloride solution (0.1 M) (80 µl) in IV tube, distilled water (80 µl) in V tube, serum (160 µl) and lysates of lymphocytes and erythrocytes (40 µl) in III, IV and V tubes, AMP solution (10 mM) (80 µl) in III and IV tubes followed by mixing and incubation for 30 minutes at 37˚C in water bath. Then 11% TCA (0.68 M*)* (800 µl) was added followed by centrifugation at 5340 rpm (3000 × g) in refrigerated centrifuge (HermleLabortechnik GmbH Germany Type Z 36 HK) using angular rotor (no. 8) at 10˚C for 5 minutes and supernatant (1000 µl) transferred in properly labelled respective test tubes. Then KH_2PO_4 standard (50 µg/ml) (60 µl *i.e*. 3 µg) was pulsed in II, III and IV test tube followed by pipetting of 3000 µl distilled water into I and V and 2940 µl into II, III and IV tubes, molybdate solution (40 mM) (800 µl), reducing agent (400 µl) added successively into each test tube and mixed and allowed to stand for 10 minutes followed by addition of acetate solution (2.5 M) (1600 µl) and distilled water (1200 µl) into each tube. The reaction was allowed to stand for 5 minutes followed by measurement of O.D. at 578 nm, using UV-Vis spectrophotometer (Cary Bio 100 Varian). The enzyme activity was expressed in U/l in case of serum, mU/million lymphocytes, and mU/billion RBC.

2.7. Adenosine Deaminase (ADA) and Its Isoenzymes Assay

Total adenosine deaminase (ADA) activity was determined by the method of Giusti [40] and its isoenzymes (ADA1, ADA2) activities were determined as described by Goodarzi *et al.* [41].

2.7.1. Adenosine Deaminase (ADA) Assay

Four test tubes were prepared and labelled as reagent blank, standard, control and sample and followed by pipetting in test tubes: phosphate buffer (50 mM NaH_2PO_4 and $Na_2HPO_4{\cdot}12H_2O$, pH 6.5) (420 µl) in reagent blank tube, ammonium sulphate standard solution (75 µM) (400 µl) and distilled water (20 µl) in standard tubes, buffered adenosine solution (21 mM adenosine, 50 mM phosphate, pH 6.5) (380 µl in serum and erythrocytes labelled tubes and 410 µl in lymphocytes labelled tube) in sample and control tubes, sample (40 µl serum, 40 µl erythrocyte lysate and 10 µl of lymphocyte lysate) in sample tube followed by thorough mixing and incubation for 60 minutes at 37˚C in water bath. Phenol-nitroprusside solution (106 mM phenol, 0.17 mM sodium nitroprusside) (1200 µl) and alkaline hypochlorite solution (11 mM NaOCl, 125 mM NaOH) (1200 µl) in all the four test tubes and sample (40 µl serum, 40 µl erythrocyte lysate and 10 µl lymphocyte lysate) were added in control tube successively. The contents of the tube were mixed before pipetting into the next test tube followed by incubation for 30 minutes at 37˚C in water bath. The O.D. was measured against distilled water at 630 nm, using UV Visible spectrophotometer (Cary Bio 100 Varian). Enzyme activity was expressed in U/l in case of serum, mU/million lymphocytes, and mU/billion RBC.

2.7.2. Adenosine Deaminase Isoenzymes (ADA1, ADA2) Assay

ADA isoenzymes were distinguished by their differential inhibition by EHNA[Erythro-9(2-hydroxy-3-nonyl) adenine], which is known to inhibit ADA1. Four test tubes were prepared and labelled as reagent blank, standard, control and sample and followed by pipetting in test tubes: phosphate buffer (50 mM NaH_2PO_4 and $Na_2HPO_4{\cdot}12H_2O$, pH 6.5) (420 µl) in reagent blank tube, ammonium sulphate standard solution (75 µM) (400 µl) and distilled water (20 µl) in standard tubes, buffered adenosine solution (42 mM adenosine, 50 mM phosphate, pH 6.5) (200 µl in serum, lymphocytes and erythrocytes labelled tubes) in sample and control tubes, EHNA solution (1 mM) (84 µl), sample (40 µl serum, 40 µl erythrocyte lysate and 10 µl of lymphocyte lysate) in sample tube and

phosphate buffer (50 mM NaH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$, pH 6.5) (96 µl in serum and erythrocyte labelled and 126 µl in lymphocyte labelled tubes) to make up the total volume equal (420 µl) followed by thorough mixing and incubation for 60 minutes at 37˚C in water bath. Phenol-nitroprusside solution (106 mM phenol, 0.17 mM sodium nitroprusside) (1200 µl) and alkaline hypochlorite solution (11 mM NaOCl, 125 mM NaOH) (1200 µl) in all the four test tubes and sample (40 µl serum, 40 µl erythrocyte lysate and 10 µl lymphocyte lysate) were added in control tube successively. The contents of the tube were mixed before pipetting into the next test tube followed by incubation for 30 minutes at 37˚C in water bath. The O.D. was measured against distilled water at wavelength 630 nm, using UV-V is spectrophotometer (Cary Bio 100 Varian). The ADA2 enzyme activity was expressed in U/l in case of serum, mU/million lymphocytes, and mU/billion RBC. ADA_1 activity was calculated by subtracting ADA_2 from total ADA activity.

2.8. Statistical Analysis

One way analysis of variance (ANOVA) and Tukey HSD test was applied for statistical analysis. The Pearson's test was used to determine correlation. Normally distributed data is displayed as mean and 95% confidence interval (CI). The p value of <0.05 was considered significant.

3. Results

3.1. Characteristics of Study Population

The characteristics of the study population are shown in **Table 1**.

Table 1. The clinicophysiological details of subjects.

Subject information parameters	Healthy controls	Bronchial asthma groups		
		Mild persistent (Group I)	Moderate persistent (Group II)	Severe persistent (Group III)
Age (years)	31.13 ± 9.79	31.93 ± 8.97	35.93 ± 10.46	34.53 ± 11.38
(Range)	(19 - 52)	(18 - 48)	(20 - 54)	(19 - 53)
Sex				
Male	6	4	8	8
Female	9	11	7	7
Duration of illness	-	1.60 ± 1.52	3.52 ± 3.30	5.33 ± 3.27
(Range)	-	(2 months - 6 years)	(6 months - 10 years)	(1 - 11 years)
Family history of asthma				
Positive	None	2	2	1
Absent		13	13	14
Diet				
Vegetarian	7	8	8	6
Non-vegetarian	8	7	7	9
FEV1% predicted	-	84.20 ± 3.28	71.40 ± 6.16	51.0 ± 5.75
(Range)		(80 - 89)	(61 - 78)	(36 - 57)
FEV1/FVC% predicted	-	88.60 ± 7.87	84.66 ± 10.8	69.26 ± 10.79
(Range)		(80.73 - 96.47)	(73.86 - 95.46)	(58.47 - 80.05)

Parameter values are mean ± S.D for n = 15 subjects. FEV1: forced expiratory volume in 1 sec.

3.2. Characteristics of Isolated Lymphocytes and Erythrocytes

Total number of lymphocytes obtained from peripheral blood of controls was 1.46 ± 0.03 (mean ± SEM) million/ml and of asthmatic patients was 1.79 ± 0.04 (mean ± SEM) million cells/ml. The differential cell counts of the isolated lymphocytes from blood revealed that 96.0% ± 0.45% (mean ± SEM) of the cells were lymphocytes in the cell suspension. The viability of the cells was 96.00% ± 0.097% (mean ± SEM). Total number of erythrocytes obtained from peripheral blood was 6.00 ± 0.12 (mean ± SEM) billion cells/ml in normal subjects and 5.51 ± 0.09 (mean ± SEM) billion/ml in asthma.

3.3. Adenosine Levels in Healthy Controls and Asthmatics

In asthma, adenosine levels in serum, lymphocytes and erythrocytes were found to be raised significantly as compared to healthy controls ($p < 0.0001$). The difference was statistically significant between control and mild persistent [($p < 0.001$) in serum and ($p < 0.0001$) in lymphocytes and erythrocytes], control and moderate persistent ($p < 0.0001$) and control and severe persistent asthmatics ($p < 0.0001$) in serum, lymphocytes and erythrocytes separately. Within the three groups of asthmatics, difference in adenosine levels were significant between mild persistent and severe persistent asthmatics in serum, lymphocytes and erythrocytes ($p < 0.05$). The difference between mild and moderate persistent and between moderate and severe persistent was found to be significant in lymphocytes ($p < 0.05$) and not significant in serum and erythrocytes (**Table 2**). The FEV1% was observed to have a negative correlation with the serum adenosine levels ($r = -0.4879$, $p < 0.001$) (**Figure 1(a)**), adenosine levels of lymphocytes ($r = -0.7097$, $p < 0.0001$) (**Figure 1(b)**), and with the erythrocytes' adenosine levels ($r = -0.5581$, $p < 0.0001$) (**Figure 1(c)**).

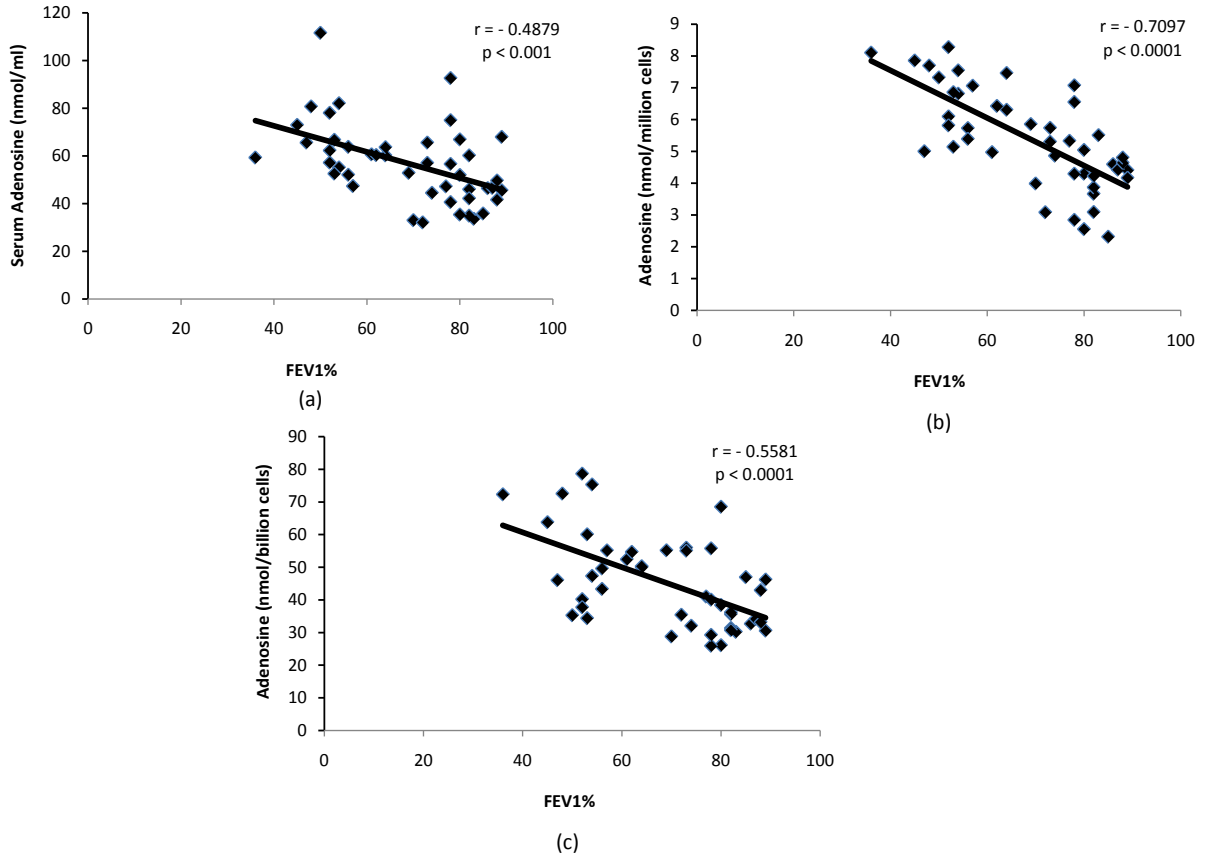

Figure 1. Relationship between FEV1% predicted and adenosine levels in (a) serum expressed as nanomoles/ml (b) lymphocytes expressed as nanomoles/million cells and (c) erythrocytes expressed as nanomoles/billion cells of asthmatic patients. FEV1: forced expiratory volume in one second.

Table 2. Adenosine levels in serum, lymphocytes and erythrocytes.

	Healthy controls	Bronchial asthma groups		
		Mild persistent (Group I)	Moderate persistent (Group II)	Severe persistent (Group III)
Serum[a]	27.82 ± 1.90	47.01 ± 2.83**	56.14 ± 4.06***	67.17 ± 4.20***†
(Range)	(16.61 - 41.83)	(33.62 - 67.98)	(32.11 - 92.63)	(47.36 - 111.60)
Lymphocytes[b]	2.54 ± 0.14	4.13 ± 0.23***	5.34 ± 0.35***†	6.71 ± 0.28***†‡
(Range)	(1.14 - 3.25)	(2.31 - 5.50)	(2.84 - 7.46)	(5.00 - 8.27)
Erythrocytes[c]	20.31 ± 1.40	37.57 ± 2.70***	44.11 ± 2.93***	54.11 ± 3.97***†
(Range)	(9.96 - 29.58)	(26.07 - 68.51)	(25.92 - 55.95)	(34.36 - 78.65)

[a]Expressed as nanomoles/ml (mean ± SEM). [b]Expressed as nanomoles/million lymphocytes (mean ± SEM). [c]Expressed as nanomoles/billion erythrocytes (mean ± SEM). $^{**}p < 0.001$, $^{***}p < 0.0001$ in comparison with healthy controls. $^{\dagger}p < 0.05$ in comparison with Group I, $^{\ddagger}p < 0.05$ in comparison with Group II. N = 15 in each group.

3.4. 5′-Nucleotidase Activity in Healthy Controls and Asthma Patients

In asthma, the 5′-NT activity was raised significantly in moderate persistent [($p < 0.0001$) in serum and ($p < 0.05$) in lymphocytes], and severe persistent [($p < 0.0001$) in serum and lymphocytes and ($p < 0.05$) in erythrocytes] asthmatics in comparison with the healthy controls but the difference was not significant statistically between mild persistent and healthy controls in serum, lymphocytes and erythrocytes. The difference was significant between mild and severe persistent asthmatics in serum and lymphocytes ($p < 0.05$) and between mild and moderate ($p < 0.05$) and between moderate and severe persistent ($p < 0.05$) asthmatics in lymphocytes (**Table 3**). Comparison among various groups of bronchial asthma revealed no statistically significant difference in erythrocytes. The FEV1% showed a negative correlation with the serum 5′-NT activity ($r = -0.4018$, $p < 0.05$) (**Figure 2(a)**) and with the 5′-NT activity in lymphocytes ($r = -0.8362$, $p < 0.0001$) (**Figure 2(b)**). No correlation between FEV1% and 5′-NT activity in erythrocytes ($r = -0.1399$) existed (**Figure 2(c)**).

3.5. Adenosine Deaminase and Its Isoenzymes Activity in Healthy Controls and Asthmatics

Total ADA and its isoenzymes (ADA1, ADA2) activities were assayed in serum, lymphocytes and erythrocytes (**Table 4**). In asthma, the total activity of ADA, ADA1 and ADA2 in serum decreased as compared to healthy controls. The difference was significant between control and moderate persistent asthmatics [($p < 0.0001$) for total ADA and ADA1, ($p < 0.001$) for ADA2], control and severe persistent asthmatics [($p < 0.0001$) for total ADA, ADA1 and ADA2]. However, the difference was not significant between mild persistent and healthy controls. Further, the decrease in total ADA, ADA1 and ADA2 was significant between mild and moderate persistent asthmatics and between mild and severe persistent asthmatics ($p < 0.05$). The FEV1% had a positive correlation with the serum total ADA ($r = 0.6127$, $p < 0.0001$), ADA1 ($r = 0.4630$, $p < 0.001$) and ADA2 ($r = 0.5804$, $p < 0.0001$) activity (**Figure 3**).

In lymphocytes, the total ADA and ADA1 activity decreased significantly in moderate persistent and severe persistent asthmatics in comparison with healthy controls ($p < 0.0001$). The difference was not significant between mild persistent asthmatics and healthy controls. Also, no significant difference in ADA2 activity was found between asthmatics and healthy controls. Comparison among bronchial asthma groups revealed significant decrease in total ADA and ADA1 in moderate persistent than in mild persistent and significant decrease in severe persistent than in moderate persistent and mild persistent ($p < 0.05$) (**Table 4**). There was a positive correlation ($r = 0.7015$, $p < 0.0001$) between FEV1 (% predicted) and total ADA activity (**Figure 4(a)**).

In erythrocytes, the total ADA, ADA1 and ADA2 activity in erythrocytes of bronchial asthma patients increased significantly in mild persistent asthmatics as compared to healthy controls ($p < 0.0001$), but became normal in moderate and severe persistent (**Table 4**). The difference was significant between mild and moderate persistent and between mild and severe persistent ($p < 0.0001$) but not between moderate and severe persistent asthmatics. A positive correlation ($r = 0.5473$, $p < 0.0001$) was observed between FEV1 (% predicted) and total ADA activity (**Figure 4(b)**).

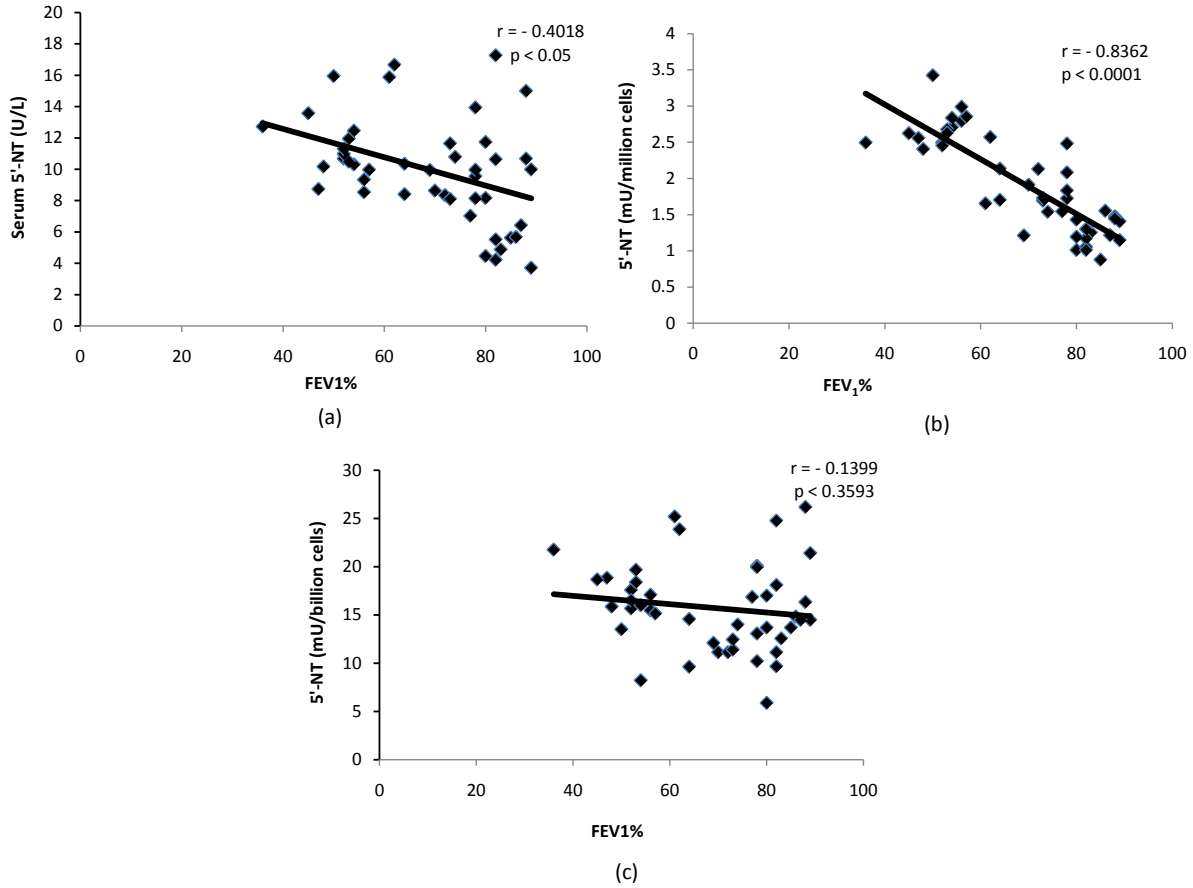

Figure 2. Correlation between FEV1% predicted and 5′-NT activity in (a) serum expressed as Units/l (b) lymphocytes expressed as milliunits/million cells and (c) erythrocytes expressed as milliunits/billion cells of asthmatic patients. FEV1: forced expiratory volume in one second. 5′-NT: 5′-nucleotidase.

Table 3. 5′-nucleotidase (5′-NT) activity in serum, lymphocytes and erythrocytes.

	Healthy controls	Bronchial asthma groups		
		Mild persistent (Group I)	Moderate persistent (Group II)	Severe persistent (Group III)
Serum[a]	6.26 ± 0.26	8.26 ± 1.07	10.49 ± 0.75***	11.14 ± 0.50***†
(Range)	(4.28 - 7.63)	(3.70 - 17.27)	(7.02 - 16.66)	(8.53 - 15.94)
Lymphocytes[b]	1.53 ± 0.08	1.23 ± 0.05	1.86 ± 0.09*†	2.69 ± 0.07***†‡
(Range)	(0.98 - 1.97)	(0.88 - 1.55)	(1.21 - 2.57)	(2.40 - 3.42)
Erythrocytes[c]	11.75 ± 0.68	15.60 ± 1.39	15.03 ± 1.29	16.54 ± 0.80*
(Range)	(7.49 - 15.48)	(5.86 - 26.17)	(9.61 - 25.18)	(8.20 - 21.74)

[a]Expressed as U/L (mean ± SEM). [b]Expressed as mU/million lymphocytes (mean ± SEM). [c]Expressed as mU/billion erythrocytes (mean ± SEM). $^{*}p < 0.05$, $^{***}p < 0.0001$ in comparison with healthy controls, $^{\dagger}p < 0.05$ in comparison with Group I, $^{\ddagger}p < 0.05$ in comparison with Group II. n= 15 in each group.

4. Discussion

Adenosine is a signalling molecule produced as a result of cell stress or damage [26]. Several studies demonstrate elevated adenosine levels in patients with chronic lung disease [42]. Adenosine levels are elevated in

Table 4. ADA and its isoenzymes activities.

	Healthy controls	Bronchial asthma groups		
		Mild persistent (Group I)	Moderate persistent (Group II)	Severe persistent (Group III)
Serum				
ADA[a]	11.16 ± 0.39	10.69 ± 0.32	8.29 ± 0.31***†	7.71 ± 0.33***†
(Range)	(8.82 - 14.37)	(7.91 - 20.4)	(6.46 - 10.57)	(8.53 - 15.94)
ADA1[a]	3.77 ± 0.20	3.53 ± 0.23	2.24 ± 0.16***†	2.43 ± 0.18***†
(Range)	(2.91 - 5.85)	(1.77 - 4.76)	(1.31 - 3.61)	(1.16 - 3.46)
ADA2[a]	7.39 ± 0.29	7.16 ± 0.19	6.06 ± 0.24**†	5.28 ± 0.23***†
(Range)	(5.58 - 9.48)	(6.14 - 8.56)	(5.07 - 8.42)	(3.36 - 6.38)
Lymphocytes				
ADA[b]	6.91 ± 0.19	7.39 ± 0.19	5.57 ± 0.29***†	4.53 ± 0.21***†‡
(Range)	(5.58 - 7.95)	(5.79 - 8.24)	(4.07 - 7.46)	(2.88 - 5.79)
ADA1[b]	6.45 ± 0.17	6.84 ± 0.19	5.10 ± 0.25***†	4.21 ± 0.19***†‡
(Range)	(5.30 - 7.32)	(5.26 - 7.89)	(3.74 - 6.70)	(2.65 - 5.47)
ADA2[b]	0.46 ± 0.05	0.55 ± 0.05	0.47 ± 0.05	0.32 ± 0.03†
(Range)	(0.20 - 0.86)	(0.35 - 0.79)	(0.19 - 0.76)	(0.12 - 0.49)
Erythrocytes				
ADA[c]	3.77 ± 0.23	6.55 ± 0.27***	3.74 ± 0.27†††	3.71 ± 0.34†††
(Range)	(2.02 - 5.09)	(4.52 - 8.28)	(1.44 - 5.22)	(2.88 - 5.79)
ADA1[c]	3.60 ± 0.23	6.24 ± 0.26***	3.57 ± 0.26†††	3.65 ± 0.33†††
(Range)	(1.87 - 4.98)	(4.34 - 8.01)	(1.37 - 5.00)	(0.98 - 5.76)
ADA2[c]	0.17 ± 0.01	0.31 ± 0.03***	0.17± 0.01†††	0.14 ± 0.01†††
(Range)	(0.11 - 0.27)	(0.14 - 0.60)	(0.07 - 0.27)	(0.12 - 0.49)

[a]Expressed as U/L (mean ± SEM). [b]Expressed as mU/million lymphocytes (mean ± SEM). [c]Expressed as mU/billion erythrocytes (mean ± SEM). $^{**}p < 0.001$, $^{***}p < 0.0001$ in comparison with healthy controls, $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.0001$ in comparison with Group I, $^{\ddagger}p < 0.05$ in comparison with Group II. n = 15 in each group.

lavage fluid collected from asthmatics [21], in the exhaled breath condensate of patients with allergic asthma [43], in plasma of asthmatic subjects following bronchial provocation with allergen [44] and in patients with exercise-induced asthma [45]. Adenosine levels are controlled by the rates of adenosine biosynthesis and its catabolism. Adenosine is generated intracellularly and released through constitutively expressed nucleoside transporters. Extracellular adenosine is formed from the dephosphorylation of adenine nucleotides, a reaction catalysed by 5′-nucleotidase. Once produced, adenosine can engage cell surface adenosine receptors or be removed by metabolism to inosine by adenosine deaminase (ADA).

The 5′-nucleotidase (CD73) is the major enzyme for extracellular adenosine production [42]. 5′-nucleotidase levels are up-regulated in the lungs of mouse models with chronic lung disease including ADA-deficient mice and mice exposed to bleomycin [46]. In addition, bronchial epithelial cells from patients with cystic fibrosis exhibit increased CD73 activity [47]. These findings suggest that the up-regulation of 5′-nucleotidase is an important purinergic remodelling response in environments where adenosine has been shown to regulate the disease. In the current study, we observed that the enzymatic activity of 5′-nucleotidase was significantly increased in

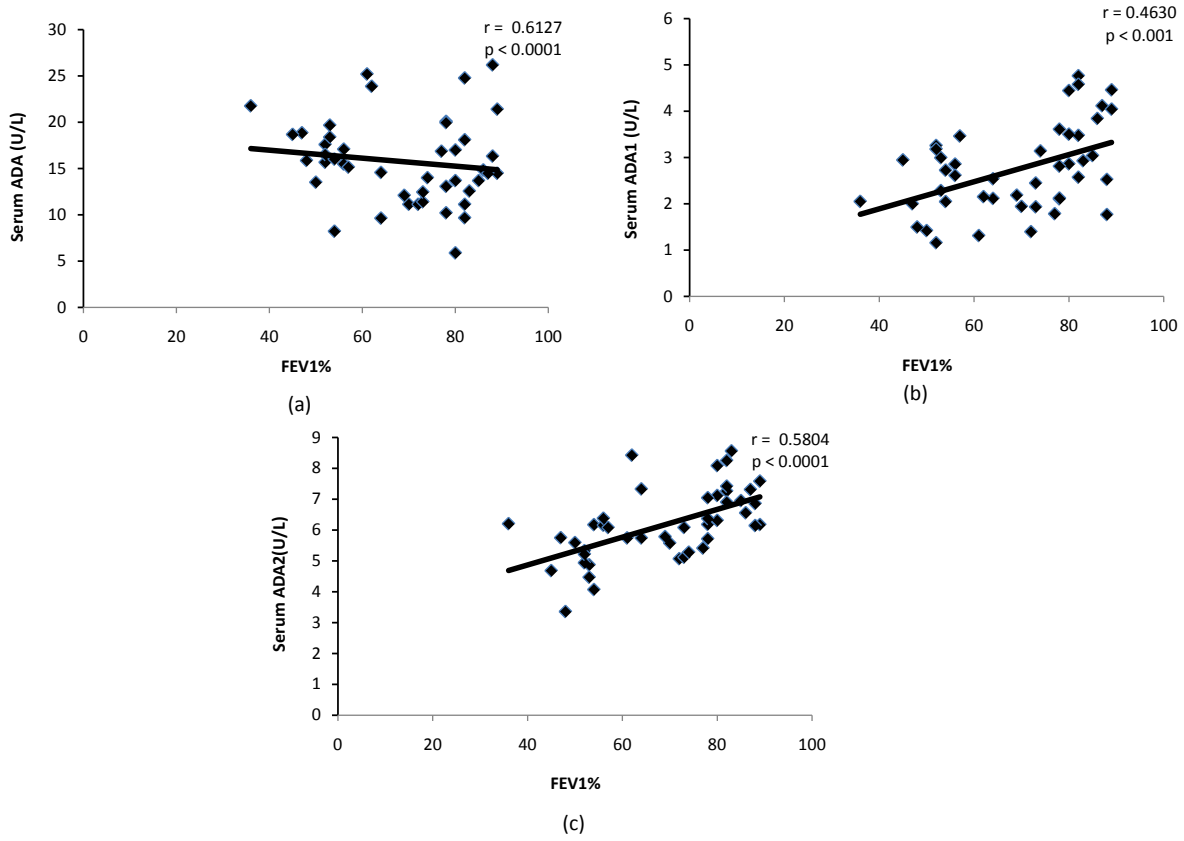

Figure 3. Relationship between FEV1% predicted and serum (a) total ADA activity (b) ADA1 and (c) ADA2 activity expressed as Units/l in asthma. FEV1: forced expiratory volume in one second. ADA: Adenosine deaminase.

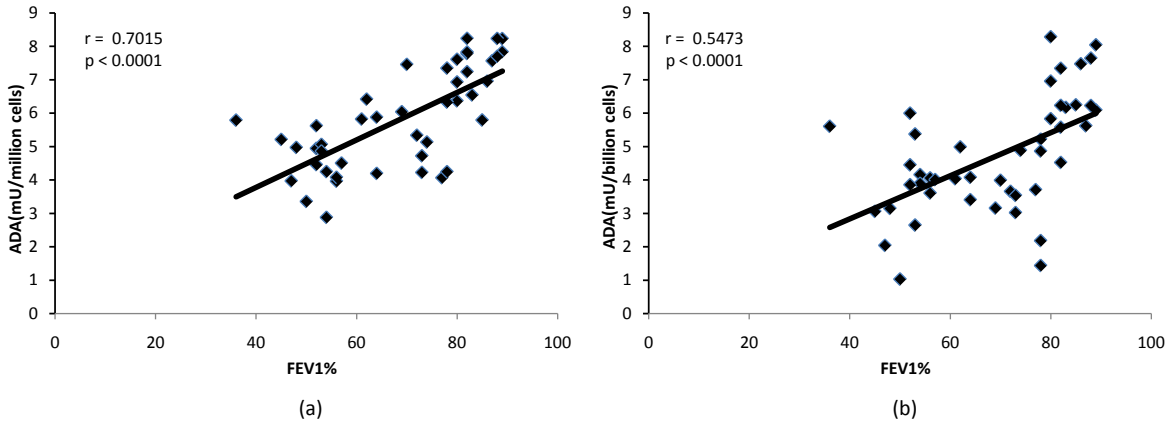

Figure 4. Correlation between FEV1% predicted and ADA activity in (a) lymphocytes expressed as milliunits/million cells and (b) erythrocytes expressed as milliunits/billion cells of asthmatic patients. FEV1: forced expiratory volume in one second. ADA: Adenosine deaminase.

serum as well as lymphocytes of both moderate persistent (Group II) and severe persistent asthma (Group III) groups ($p < 0.0001$). Consistent with this, adenosine levels in serum and lymphocytes in asthma patients were found to be raised significantly compared with subjects with preserved lung function. Further, in this study, the changes in 5′-nucleotidase activity in serum and lymphocytes showed a significant reciprocal correlation with FEV1 of asthma patients suggesting that as the severity of airway obstruction increases (shown by decrease in

FEV1), the level of 5′-nucleotidase activity was found to be increased. Similarly, raised adenosine levels in serum and lymphocytes showed a significant inverse correlation with FEV1 of asthma patients which suggest that with the increase in the severity of airway obstruction, levels of adenosine were found to be elevated. These findings suggest that 5′-nucleotidase enzyme might play a major role not only in the production of adenosine but also in the modulation of adenosine levels in serum and lymphocytes and the up-regulation of 5′-nucleotidase might be considered as an important purinergic response in bronchial asthma. In case of erythrocytes, adenosine levels were found to be increased in asthma patients but 5′-nucleotidase activity was raised only in severe persistent group and as no correlation was found between severity of asthma and 5′-nucleotidase activity, therefore it could be assumed that 5′-nucleotidase contributes little in regulating levels of adenosine in erythrocytes. These findings suggest that there is an increased production of adenosine in asthma and the increase in 5′-nucleotidase in serum and lymphocytes provides a novel and important means of determining the capacity of adenosine generation.

Adenosine deaminase (ADA) is the major enzyme of adenosine metabolism [48]. It deaminates adenosine to inosine. Elevated levels of adenosine have been shown to be associated with features of inflammation, alveolar remodelling, pulmonary fibrosis, and mucus secretion in lungs of a mice model over-expressing the Th2 cytokines viz. IL-4 or IL-13 [49] [50]. Interestingly, ADA transcripts and enzymatic activity are selectively down-regulated in the lungs of these mice suggesting a purinergic remodelling response directed at promoting adenosine accumulation. In our study, we observed that the total activity of adenosine deaminase was significantly decreased in serum of asthma patients. Total ADA activity decreased in all the three groups of asthmatics, which was however decreased significantly in moderate (Group II) and severe persistent (Group III) groups. The decrease in total ADA activity in serum of asthma patients was apparently primarily due to low ADA2 activity, although ADA1 activity also decreased in moderate and severe persistent groups. Further, a significant positive correlation between FEV1 of asthma patients and reduction in the total activity of ADA and its isoenzymes in serum indicates that with the increase in the severity of airway obstruction, activities of ADA and its isoenzymes (ADA1 and ADA2) decreased significantly or *vice versa*. In lymphocytes of asthma patients, total ADA activity was found to be decreased as compared to subjects with preserved pulmonary function in our study. The decrease in activity was found to be significant in moderate persistent (Group II) and severe persistent (Group III) asthma patients and no significant alteration in its activity was found in mild persistent (Group I) group. The decreased total ADA activity in bronchial asthma was largely due to reduced activity of the ADA isoenzyme ADA1 and the contribution of ADA2 was only minor. Further, fall in the activity of ADA and its isoenzymes in lymphocytes showed a significant positive correlation with FEV1 of asthma patients suggesting that with the increase in the severity of airway obstruction, activities of ADA and its isoenzyme (ADA1) were decreased significantly. Thus, fall in total activity of ADA along with raised adenosine levels in serum and lymphocytes and positive correlation of both with the severity of airway obstruction indicates that ADA enzyme might also act as a major regulator of adenosine levels in serum and lymphocytes of asthma patients. In case of erythrocytes, total ADA activity was found to be raised significantly only in mild persistent (Group I) group of asthma but the difference was not significant in moderate(Group II) and severe persistent (Group III) group when compared to healthy control. This increased activity of ADA was predominantly due to increased activity of the ADA isoenzyme, ADA1. ADA and its isoenzymes activities in erythrocytes showed a significant positive correlation with FEV1 of asthma patients. These findings suggest that determination of ADA, ADA1 and ADA2 in serum, and ADA or more specifically ADA1 in lymphocytes, might serve as a way of determining the capacity of adenosine generation.

Increased activity of 5′-nucleotidase and concomitant low activity of ADA along with raised levels of adenosine in asthma patients suggest that these two enzymes might play a crucial role in controlling levels of adenosine in serum. Elevation in serum adenosine level that was found in this study may lead to increased circulation of adenosine in lungs which may also reflect its increased level in lung of asthma patients (**Figure 5**). It is also known that the leakage of metabolites and enzymes from a tissue may cause an increase in the blood and may thus reflect the changes in a particular tissue in a particular condition. In the setting of tissue injury, the predominant source of extracellular adenosine arises from the breakdown of adenine nucleotides [51] [52]. Elevations in extracellular adenosine can result from either an increase in intracellular adenosine followed by its release into the extracellular space, or by the release of adenine nucleotides followed by their extracellular catabolism into adenosine [53]. Intracellularly, adenosine can be generated from the dephosphorylation of AMP by cytosolic 5′-nucleotidases [54]. Extracellularly, adenosine can be generated following the release and dephosphorylation

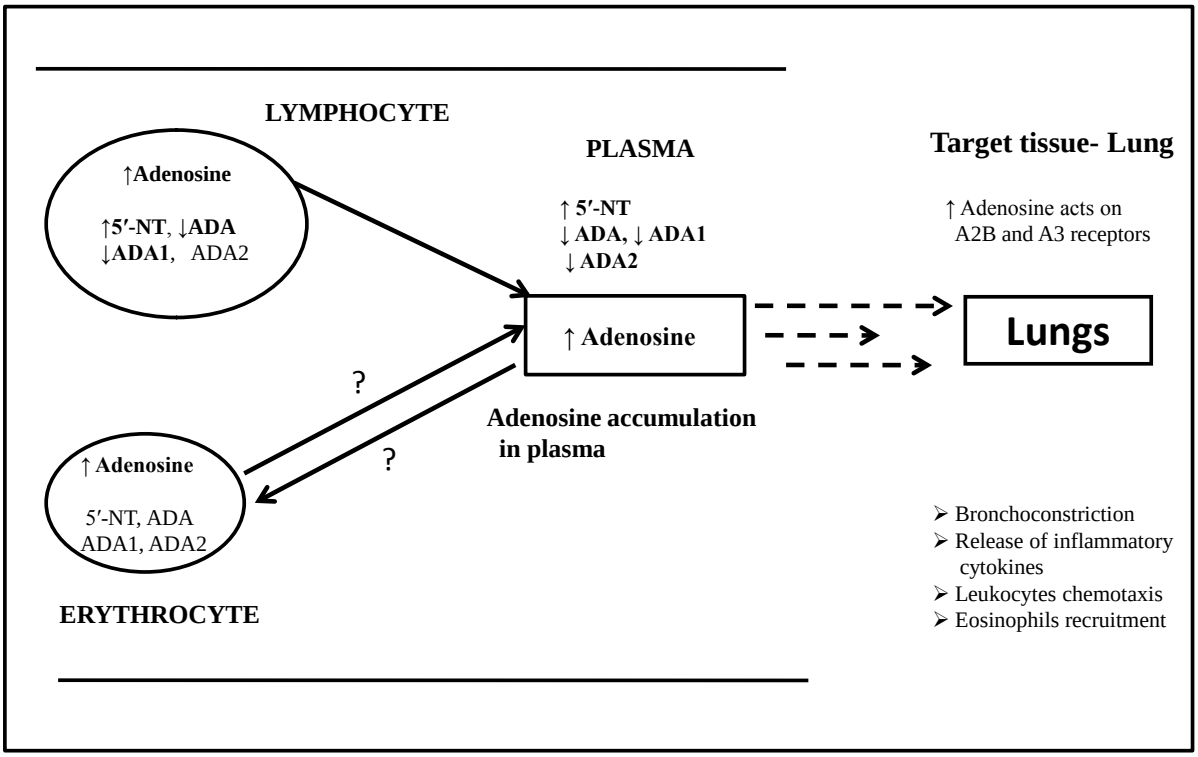

Figure 5. Adenosine metabolism in bronchial asthma. In asthma, elevated levels of adenosine in plasma are found due to (a) its increased synthesis extracellularly as a result of increased 5′-NT activity and decreased total ADA, ADA1 and ADA2 isoenzyme activity, (b) increased synthesis in lymphocytes (because of increased 5′-NT activity and decreased total ADA and ADA1 isoenzyme activity) followed by its release into plasma. In erythrocytes, adenosine levels were found to be raised but no change in 5′-NT and total ADA and its isoenzymes' activity were found. Thus, role of erythrocytes in adenosine release into plasma or adenosine uptake from plasma and storage is uncertain. Adenosine, thus accumulated in plasma may lead to its increased circulation in lungs, where it acts via its receptors to cause inflammatory changes.

of adenine nucleotides [53]. ATP and AMP are released from activated granulocytes [55] [56], upon degranulation [57]. Bronchial epithelium can release ATP under basal conditions [58] and upon perturbation of the plasma membrane [59]. Extracellular ATP and ADP can be converted into AMP and then into adenosine by ecto-5′-nucleotidase (CD73). In addition to the release of adenine nucleotides from cells, there is also evidence that adenosine itself can be released from inflammatory mast cells during the degranulation process [60]. This highlights the importance of inflammatory cells as a direct source of adenosine. It is still unclear which pathway and which enzymes are responsible for extracellular adenosine accumulations following tissue injury. But, from our study, it may be proposed that the increased level of adenosine in serum may be due to its leakage from airways or from lymphocytes and erythrocytes in asthma. Further, adenosine accumulation in the lung is not only a product of lung inflammation and damage, but can directly affect signalling pathway that lead to features of chronic lung disease [61]. Adenosine elevations in the lungs of patients with asthma suggest that this signalling molecule may regulate bronchoconstriction and the unique bronchial sensitivity of asthmatics to adenosine suggest a fundamental alteration in adenosine receptor signalling in the lungs of these patients. Adenosine, via engagement of the A2BR, increases the release of IL-6 and monocyte chemotactic protein-1 from bronchial smooth muscle cells [62] providing a mechanism whereby adenosine acts as a pro-inflammatory mediator in the airway. Recently, Ethier *et al.* found that the A1R mediates mobilization of calcium in human bronchial smooth muscle cells, suggesting that adenosine has direct effects on contractile signalling pathways [63], which may be one of the mechanisms for bronchoconstriction. Activation of the A2BR on human lung fibroblasts increases the release of IL-6 and induces differentiation into myofibroblasts suggesting that adenosine, via A2BR participates in the remodelling process occurring in chronic lung diseases [64]. Chunn *et al.* revealed that controlling adenosine levels with the use of exogenous ADA treatments may provide a significant approach to seize the progression or alter the features of pulmonary fibrosis in severe asthma [65].

5. Conclusion

The present study clearly demonstrates that the adenosine levels are raised in serum, lymphocytes and erythrocytes in bronchial asthma patients and correlation of this increase in adenosine with increase in airway obstruction provides evidence in favour of adenosine for its role as a crucial inflammatory mediator in asthma. It also confirms that adenosine levels tend to increase in serum, lymphocytes and erythrocytes with the severity of bronchial asthma. Next, increase in activity of 5′-nucleotidase and a concomitant decrease in activity of adenosine deaminase and its isoenzymes in serum and lymphocytes demonstrate the importance of these two enzymes in adenosine metabolism and suggest that the balance between these two enzymes determines the levels of adenosine in serum and lymphocytes which might act as potential inflammatory mediator in asthma. Further, increase in activity of 5′-nucleotidase and a corresponding decrease in adenosine deaminase activity with the worsening of asthma clearly emphasize that these two enzymes play a crucial role in accumulation of adenosine which may result in pathogenesis of bronchial asthma, or *vice versa*.

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