Granulocyte-Macrophage colony stimulating factor in asthmatic patients infected with respiratory syncytial virus

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ABSTRACT
Introduction: It is estimated that at least 30 to 40% of asthma attacks in adults are related to respiratory infections with viruses. The majority of asthma-related viruses include respiratory syncyial virus (RSV), rhinovirus, and parainfluenza. Inflammatory cytokines are supposed to play a vital role in causing inflammation of the respiratory tract as regulators of proliferation, chemotaxis, and activation of inflammatory cells.

Objectives: The aim of this study is to assess the role of Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) in asthmatic airway hyper-responsiveness associated with RSV infections.

Materials and Methods: Forty five asthmatic cases and 45 healthy individuals were studied in a cross-sectional design. All asthmatics underwent symptom score assessment. GM-CSF concentrations in sputum and RSV-IgM/IgG in serum samples were measured for all participants by Enzyme Linked Immuno-Sorbent Assay (ELISA).

Results: The GM-CSF concentration level was significantly higher in asthmatics (270.27±194.87 pg/mL) especially among moderate and severe disease with mean concentration of 197.33±98.47 and 521.08±310.04 respectively, compared to healthy controls (22.20±21.27 pg/mL) (p=0.0001). The sputum level of GM-CSF in asthmatics is highly significant associated with positive anti-RSV IgG sera which represents 35/45 (77.8%) with mean GM-CSF concentration of (276.99±86.42) compared with controls at about 31/45 (68.9%) with mean GM-CSF mean concentration of (22.84±23.47). On the other hand, positive anti-RSV IgM in asthma cases was 8 out of 45 (17.8%) with GM-CSF mean concentration of (307.25±306.65). Furthermore, GM-CSF sputum level was significantly correlated with eosinophil count especially in moderate and severe asthma.

Conclusions: This study revealed that GM-CSF level is associated with eosinophilia and indicates asthma severity that might be evident during RSV infection. The distinctive GM-CSF features observed in the sputum from asthmatics with RSV may be useful as a diagnostic method to help match patients with antibody therapy.

KEYWORDS:
Asthma, GM-CSF, eosinophil, cytokines, RSV, ELISA

INTRODUCTION
Asthma is known as an inflammatory disease in which a variety of cytokines are implicated. Thus, in episodes of asthma, numerous cytokines, consist of TNF, GM-CSF, IL1β, IL2, IL6, PG-D2 and Periostin are detectable in blood, sputum and in bronchio-alveolar lavage fluids. Among these, granulocyte-macrophage colony-stimulating factor (GM-CSF) is recognized to have a vital role in eosinophil survival and in the activation of Antigen Presenting Cells (APC).

GM-CSF is a glycoprotein of monomeric type produced by many cells including macrophages, T- cells, mast cells, endothelial cells and fibroblasts that were primarily regarded as (haematopoietic growth factor). It is stated at present that GM-CSF is a cytokine that responsible to activate, differentiate and plays a role in both adaptive and innate immune elements survival including cells of granulocytes, macrophages, dendrocytes and lymphocytes. Normal lung epithelium secrete small quantities of GM-CSF. However, it produced in greater amounts in asthmatics lungs epithelial cells. Epithelial cells have been shown to be the major source of GM-CSF in respiratory secretions of healthy people, while in patients with atopic diseases; eosinophils are the main and dominant source in respiratory tissues together with migrating lymphocytes and neighboring epithelial cells that are stimulated by external antigen. GM-CSF supports the development, maturation and differentiation of myeloid cell, and dendritic cell, GM-CSF formation -signaling imbalance may perhaps lead to damaging inflammatory effects. Supporting evidences have revealed that GM-CSF plays an important role in a number of inflammatory and autoimmune diseases and in response to pulmonary infections. Of note GM-CSF along with IL-3 are the major cytokines of innate immune response whose vital role is determined for the expansion and succession of atopic asthma.

Patients with asthma are prone to viral infection due to the already damaged respiratory epithelium which leads to further airflow obstruction. Furthermore, the cellular response to viral infections includes the disrupting tissue barrier and fixed inter-junctions, inhibiting apoptosis, increasing cell lysis, abnormal Th1 response and lowering the production of IFN-γ, as well as it facilitates viral receptors expression, viral shedding, but prevents viral clearance. In addition, interleukins as well as many inflammatory mediators associated with allergic reactions or remodeling cytokines are induced in response to viral infections. Viruses

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The majority of asthma-related viruses are respiratory syncytial virus (RSV), rhinovirus, and parainfluenza. RSV is a frequent viral infection of upper respiratory tract during childhood and adulthood. Though occasionally, this infection might lead to more severe type of the lower respiratory disease. Early during the period of viral infection, local cells in the airway are triggered in an independent antigen pattern, provoking anti-viral reactions but besides stimulating and conscripting cells to the airflow that may possibly cause wheezing illnesses.\textsuperscript{9} Specific orchestrated T and B cell responses against the virus may also have a worse impact in the existence of pre-inflamed respiratory airways. Lastly, virus - allergen interactions have synergistic effects that trigger bronchial inflammations.\textsuperscript{10} It is likely that the increased definition of virus mechanisms to exhort inflammation will offer therapeutic goals for treating and possibly preventing atopic diseases and asthma.\textsuperscript{11} Innate and adaptive immune system components contribute in controlling and getting rid of an infection with RSV, marked struggle against RSV re-infection that is conferred by previous infection seems to be attributed mainly through RSV-specific local secretory and serum antibodies.\textsuperscript{11}

\section*{MATERIALS AND METHODS}

\textbf{Participants:}

Forty five patients with asthma and 45 apparently healthy persons as controls were recruited in this study of a cross-sectional design; controls are of medical and paramedical healthy subjects without medical history, or any history of allergic diseases. This study was conducted at the Specialist Center for Allergy and Asthma Diseases in Baghdad, AL-Resafa and the Department of Microbiology/College of Medicine, University of Baghdad, Iraq, between July 2018 and June 2019. The protocol and methods were approved by the Scientific Ethics Committee of the Medical College Council, University of Baghdad. The approval number of ethics committee was 95 in 1 July 2018. We received verbal and written informed consent from all participants.

Patient groups were recruited randomly during their visits to seek medical care. Following respiratory infections and their impact on asthma and according to Global Initiative for Asthma (GINA) Guidelines\textsuperscript{12}, patients were divided prior to hospitalization (i.e. hospital admission as indicated for most of severe and some of moderate cases); therefore, according to their Pulmonary Function Test results that consider the force expiratory volume in one second (FEV\textsubscript{1}) ≥ 80\% as mild, from 60\%-80\% as moderate and ≤ 60\% as severe asthma. Patients complained of typical symptoms of respiratory infection such as (rhinorrhea, cough, dyspnoea, sputum, and fever) of about 4 days. However, all allergic investigations were negative for atopic diseases and those with influenza, metapneumovirus and rhinoviruses tested positive were excluded from the study.

The criteria for exclusion were chronic allergic rhinosinusitis and nasal polyps. Also, anyone who is smoker, on antibiotic treatment, on local or systemic antihistamines and/or corticosteroids was excluded.

\textbf{Sampling:}

Sampling was done under strict sterile conditions taken by patients and researcher to ensure safety measures, using personnel protective equipment (PPE) with the use a fit-tested N95 face mask. Sputum samples were spontaneously collected from clinically stable asthmatic patients who were pretreated with a short-acting beta 2 agonist (induced sputum not performed in order to overcome disease exacerbation). While an induced sputum samples in healthy controls were performed using nebulised hypertonic saline of (3\%, 4\% and 5\%) concentrations for 5-7 min, as described by Saha et al.\textsuperscript{14} The sputum was collected in a sterile container and then suspended using the 0.1\% Mucolytic Dithio-Threitol (DTT) filtered and centrifuged at 3000rpm for 20 min. The sediment was stained by Wright’s stain for differential cell counts, while the supernatants of about 2 ml were stored at −80°C as previously mentioned by Puvord et al. in 1997\textsuperscript{16} for measuring the concentration level of GM-CSF using the commercial human ELISA kit (CUSABIO. China).

Five ml of blood samples were collected in EDTA tubes from all 90 subjects centrifuged at 2000x rpm for serum separation. We placed all serum samples in a two sets of 1.5 mL Eppendorf tube each set of Eppendorf tube stored at - 20°C until used. Both sets have been used for the detection of anti RSV-IgM/IgG using 2 kits of (Demeditec Diagnostics GmbH. Germany) following the manufacturer’s instructions. GM-CSF assay utilized the quantitative sandwich enzyme immunoassay technique, and following the manufacturer’s instructions. The undiluted standard was served as the high standard (1000ng/ml) while the sample diluent was served as the zero standards (0 ng/ml). In brief, 100ul of standards, control and patient samples were added to the microwell plates, which were then incubated for 2 hours at 37°C. Next, the liquid of each microwells were removed without washing step. Then, 100ul of biotin-conjugated antibody specific for GM-CSF were added to each well, and the plates were incubated for an hour at 37°C. Next, the contents of the microwells were discarded and washed three times with a wash solution. Then, 100 ul of avidin conjugated Horseradish Peroxidase (HRP) were added to each well, and the plates were incubated again for an hour at 37°C, after that liquid was aspirated and the plate was washed 5 times. Then, the substrate solution was added and incubated for 15 minutes at 37°C, the stop solution was added to each well, and the plates were incubated for five minutes at room temperature. Finally, at 450 nm the microwell plate was read within 5 minutes, and the results were calculated. According to producer’s declaration, overall intra-assay and inter-assay precisions should be <8\% and <10\% respectively. The concentrations of GM-CSF were expressed in (pg/mL) with a range of 15.6 pg /mL - 1000 pg/mL. The minimum detectable dose of human GM-CSF was typically less than 3.9 pg/mL. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero according to manufacturer’s instructions.
The professional soft "Curve Expert 1.4" was used to make a standard curve, which was downloaded from CusBio web. A standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. The data might be linearized by plotting the log of the GM-CSF concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis.

**Statistical Analysis:**
Data were expressed as Mean ± Standard Deviation (SD), the study groups were analyzed using the t-test to compare between the mean concentrations. The strength of the correlation between different parameters were explored using the Spearman’s Rank Correlation Test. P values of ≤ 0.05 considered as statistical significant differences. We performed the analysis using the SPSS software (Statistical Package for the Social Sciences, version 20, IBM).

**RESULTS**
In this study, 45 asthmatics (22 men and 23 women, mean age 40.4 ± 12.97 years) and 45 healthy subjects (21 men and 24 women, mean age 40.01 ± 13.25 years) were included. The characteristics of the study groups shown in (Table I).

Table II shows that GM-CSF concentration level was significantly higher in asthmatics (270.27±194.87 pg/mL) especially among moderate and severe asthma with mean concentration of 197.33±198.47 and 521.08±310.04 respectively compared with control patients (22.20±21.27 pg/mL) (p=0.0001), high eosinophil count with significant differences were observed between asthmatics and control group (20.40±7.40 and 3.89±1.99) respectively with (P= 0.0001). Also illustrated in table II and III.

The mean concentrations of GM-CSF in sputum samples of asthmatic patients is highly significant associated with positive anti-RSV IgG in sera which represents 35 out of 45(77.8%) with mean GM-CSF concentration of (276.99±288.42) compared with controls at about 31of 45(68.9%) with GM-CSF mean concentration of (22.84±23.47)(t = 4.8876; two tailed P-value < 0.0001). In this study, highly significant differences were found between the eosinophil counts in the patients with RSV-IgG (20.97±7.96) compared with controls (3.94±1.77) (t= 11.6499, p=0.0001). On the other hand, positive anti-RSV IgM in asthma cases was 8 out of 45(17.8%) with GM-CSF mean concentration of (307.25±306.65). Furthermore, anti-RSV IgM were positive in 8(17.8%) asthmatic cases. On the contrary, we were unable to detect anti-RSV IgM in samples of controls (Table IV).

Interestingly, the linear relationship between the GM-CSF concentration level in sputum and eosinophil counts were illustrated in (Figure 1); a positive significant correlation between two variables were found using Pearson correlation coefficient (r= 0.456306, r² =0.2025) in asthmatics as an increase of one unit in GM-CSF sputum level will increase eosinophil count in about 20% accordingly. The two-tailed P-value equals 0.5437 and by conventional criteria, this difference is considered to be not statistically significant.

**Table I: Characteristics of the study groups**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Asthmatics (No. =45)</th>
<th>Controls (No. =45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ageyrs (Mean ± SD)</td>
<td>40.4±12.97</td>
<td>40.01±13.25</td>
</tr>
<tr>
<td>Men/Women(No.)</td>
<td>22/23</td>
<td>21/24</td>
</tr>
<tr>
<td>*FEV1: No. (%)</td>
<td>12(26.7)</td>
<td>45(100)</td>
</tr>
<tr>
<td>Normal</td>
<td>0(0)</td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>21(46.6)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>12(26.7)</td>
<td></td>
</tr>
</tbody>
</table>

* FEV1: force expiratory volume in one second.

**Table II: GM-CSF concentration among study groups**

<table>
<thead>
<tr>
<th>Study groups (No.)</th>
<th>GM-CSF Mean ±SD (pg/ml)</th>
<th>Eosinophil count Mean ±SD</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthmatics (45)</td>
<td>270.27±194.87</td>
<td>20.40±7.40</td>
<td>0.0001</td>
</tr>
<tr>
<td>Controls (45)</td>
<td>22.20±21.27</td>
<td>3.89±1.99</td>
<td></td>
</tr>
</tbody>
</table>

**Table III: Mean concentration of GM-CSF in relation to asthma severity**

<table>
<thead>
<tr>
<th>Asthmatic patients</th>
<th>No. (%)</th>
<th>GM-CSF Mean ±SD (pg/ml)</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>12(26.7)</td>
<td>65.50±65.29</td>
<td>*0.03</td>
</tr>
<tr>
<td>Moderate</td>
<td>21(46.6)</td>
<td>197.33±198.47</td>
<td>**0.0001</td>
</tr>
<tr>
<td>Severe</td>
<td>12(26.7)</td>
<td>521.08±310.04</td>
<td>***0.0001</td>
</tr>
</tbody>
</table>

*mild vs moderate,  
**moderate vs severe,  
***mild vs severe.
In this study a noninvasive specimen using sputum for studying inflammatory cytokines, being rich enough with inflammatory cells and soluble mediators of inflammation and infection; it may be used to monitor disease severity and pathology. Sputum sample collection is easily obtained from a well expectorated individuals, the problem arises in patient undergoing signs and symptoms of respiratory infection and therefore, an induced sputum collection from infected asthmatic patient might worsen his symptoms and end the patient with a crisis, so that a spontaneous expectorations from our patients were applied under close monitoring and relatively a small sputum volume were obtained. Whereas, for those healthy subjects induced sputum was used. In both groups, sputum sampling might associated with underestimation of biomarkers concentrations.

The present results demonstrated that significantly higher sputum level of GM-CSF in cases with asthma than those of controls and the level were elevated among moderate and severe asthma. These readings support a possible role of GM-CSF in bronchial asthma and indicate that the expression of GM-CSF in excess in sputum is an essential element in disease crisis as severity is elicited by previous viral infections. Our findings are supported by Saha et al. who measured GM-CSF concentration level in the sputum and bronchial cells of asthmatics and chronic obstructive pulmonary disease (COPD) and they found that GM-CSF was raised in those with moderate (7/14) and severe (11/18) in those with asthma compared to control group, suggesting that GM-CSF over-expression both in sputum and the bronchial mucosa plays a particular role in severe disease. Furthermore, Cates et al. discovered that airway hyper-responsiveness in mice can be triggered upon sensitization with intranasal dust mites via a GM-CSF arbitrated mechanism. Previous study investigated IL-6, IL-8, and GM-CSF levels and sources in the nasal secretions in response to allergen sensitization using ELISA and immunohistochemistry suggesting that strong local immune reactivity of studied proinflammatory cytokines varied according to levels, sources, and mechanisms of release but were essential in the symptom of the allergic diseases. While Sawada et al. reported that GM-CSF production during an allergic crisis may be suppressed over a long period of time by IL-4, TGF-beta, or both in a way to help control the severe allergic response, and this suppression may increase the sensitivity of inflammation in the lung tissues after the attack by affecting the resident cells maturation. Interestingly, preceding clinical studies have reported that anti-GM-CSF; blocking antibodies can be harmless and promising in many diseases of autoimmunity or inflammatory.

In this study, previous RSV infection indicated by RSV-IgG detection was common among asthmatics compared to control group suggesting prior epithelial damage. It has been reported that viral respiratory infections are the main cause of severe asthma exacerbation in 80% of cases. At least 30%

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. (%)</th>
<th>GM-CSF Mean ±SD (pg/ml)</th>
<th>Eosinophil count Mean ±SD</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Asthmatics 45 (100)</td>
<td>276.99 ± 288.42</td>
<td>20.97 ± 7.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSV-IgG 35 (77.8)</td>
<td>307.25 ± 306.65</td>
<td>18.13 ± 4.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSV-IgM 8 (17.8)</td>
<td>22.84 ± 23.47</td>
<td>3.94 ± 1.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls 45 (100)</td>
<td>22.84 ± 23.47</td>
<td>3.94 ± 1.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSV-IgG 31 (68.9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSV-IgM 0 (0)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1: Correlation of GM-CSF with eosinophil count among asthmatic patients.

Table IV: Mean GM-CSF concentrations and eosinophil counts in RSV infected study groups
- 40% of severe crisis in adult asthma are associated with respiratory viral infections and unfortunately the high-risk treatment failures are linked to these infections. Of them is RSV which can provoke acute asthma exacerbation in adults, apart from Rhinoviruses, Human Metapneumovirus, Influenza, Parainfluenza, Adenovirus, Coronavirus, and Bocavirus were all confirmed in asthmatic attacks however at low frequencies. Moreover, Johansson argued that the reasons behind this RSV induced asthma severity are not fully explained but may encounter to the immune system dysregulation towards the virus, leading to marked innate and adaptive immune elements recruitment and activation that can cause serious tissue injury. Currently no effective anti-RSV drugs or related vaccines; so infection with the virus remains of a medical concern worldwide.

Current results showed that the level of GM-CSF and eosinophilic infiltration in sputum are higher in patients with RSV infected asthma (mainly of moderate and severe type) than in uninfected patients and in healthy controls. Also the local level of eosinophilic infiltration in the respiratory tract of asthmatic patients is almost higher in seven times than in the control patients and five times higher among RSV infected asthmatics than in the controls. Previous study by Ichinohe et al. 1999 indicated that several proinflammatory mediators as GM-CSF produced following RSV infection of epithelial airways seem to contribute to eosinophil infiltration. On the other hand, Naessens et al. suggested that in post allergic attacks in the mice airways, there would be a defect in the maturation of alveolar macrophage cells residing in bronchial tree leading to a hypersensitivity of allergic lung cell to RSV infection, proposing that these immature post allergic cells might enhanced curative opportunity in controlling undesired RSV-infection with subsequent exacerbations in asthmatic patients. Again, eosinophilia is established as one of the characteristics features of asthma as a therapeutic goal in order to validate corticosteroid treatment as well as for biotherapies with monoclonal anti-ILS antibodies in patients with airway diseases. It has been recommended to modify a variety of inflammatory processes in asthma, including mucus formation, hyperplasia of smooth muscle tissues, angiogenesis, and fibrosis and thus contributing to exacerbate asthma symptoms. In fact, many biological aspects of eosinophilia are controlled by GM-CSF, including a constant development and differentiation, chemokinesis, airway hyper-responsiveness and the endurance of eosinophil during the inflammatory lung illnesses. This recruitment seems to be an organ specific for inflamed lungs. Thereby eosinophil regulation in organ specific can obtain in the context of GM-CSF signaling.

As in line with our result, other study disclosed that the sputum GM-CSF concentration was correlated with the sputum eosinophilia in subjects with asthma disease \((r_s = 0.28; p=0.007)\), all those with asthma \((r_s=0.3; p=0.04)\) and of moderate and severe disease\(^1\), indicating that the most important source of the immune mediators in patients with asthma is the stimulated eosinophils thus ensuring the growth and activation of eosinophil that have been reported also in rhinosinusitis with nasal polyp by Shin et al.\(^2\), therefore, a high abundance of eosinophils in the epithelial tissues of the patients can be explained. Thus, measuring local pro-inflammatory cytokines in sputum can be of interest to monitor the severity and to study the pathogenesis of allergic diseases.\(^3\)

**LIMITATION**
Our study has some limitations. One of the main limitations is the small sample size of 45 patients and restricted to adult asthma where most of them had experienced RSV infections in their early lives. Another limitation is that we have not been able to prove a relationship between GM-CSF and as a result of differences in treatment especially with the use of steroids as expected that the expression of sputum GM-CSF in tissues is decreased by the use of corticosteroids; this is because most patients enrolled in this study were not adherent to their treatment. Further limitation is the use of sputum sample in this study to test GM-CSF instead of broncho-alveolar levage (BAL), although later is an invasive one but measuring cytokines in the sputum of the patients may not exactly reflect the level of GM-CSF related to severity of the disease. Further studies with larger numbers of patients including childhood asthma are necessary to ascertain our findings and to detect the comparative GM-CSF expression in bronchial tissue by various types of cells.

**CONCLUSION**
These results suggesting that GM-CSF may perhaps incriminated in asthma patho-physiology and considering this cytokine as a marker of asthma exacerbation. The distinctive GM-CSF features observed in the sputum from asthmatics with RSV may be useful as a diagnostic methods to help match patients with antibody therapy. However, further studies are needed with a higher number of patients to analyze the cytokines profile in sputum for controlling allergic diseases.

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**CONFLICT OF INTEREST**
None

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**REFERENCES**