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Endostatin and Cathepsin-V in Bronchoalveolar Lavage Fluid of Patients with Pulmonary Sarcoidosis

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Abstract

Recently, it has been reported that lack of cathepsins prevent the development of lung granulomas in a mouse model of Besnier-Boeck-Schaumann (BBS) disease, sarcoidosis. There is no data about cathepsin V (Cath V) in bronchoalveolar lavage fluid (BALF) in humans. Endostatin is a novel inhibitor of lung epithelial cells. The role of this protein in BBS is not determined. The aim of this study was to evaluate the concentration of endostatin, Cath V, and IL-18 in BALF of BBS patients. We studied 22 BBS patients (Stage 2). The control group consisted of 20 healthy subjects. Cath V concentration was lower in BBS than in healthy group (16.03 ± 8.60 *vs.* 32.25 ± 21.90 pg/ml, $p = 0.004$). Both endostatin and IL-18 levels were higher in BBS than in the control group (0.88 ± 0.30 *vs.* 0.29 ± 0.04 ng/ml, $p = 0.028$; 40.37 ± 31.60 *vs.* 14.61 ± 1.30 pg/ml, $p = 0.007$, respectively). In BBS there were correlations between the levels of endostatin and IL-18 ($r = 0.74$, $p = 0.001$) as well as endostatin and DL_{CO} (diffusing capacity for carbon monoxide) ($r = -0.6$, $p = 0.013$). Receiver-operating characteristic (ROC) curves were applied to find the cut-off for the BALF levels of Cath V, endostatin, and IL-18. We conclude that Cath V and endostatin may represent an index of pulmonary sarcoidosis activity.

Keywords

Bronchoalveolar lavage fluid • Cathepsin V • Endostatin • Interelukin-18 • Sarcoidosis

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1 Introduction

Sarcoidosis is a disease of an unknown etiology. It is characterized by a chronic inflammation with creation of granulomas consisted of lymphocytes, macrophages, and epithelial cells. These cells release several chemokines and cytokines that lead to cellular proliferation. The cytokine profile of active BBS is characterized by T helper 1 (Th1) prevalence (Kieszko et al. 2007). The main cytokine involved in the polarization of T-cell response is IL-18, an interferon gamma inducing factor (Kieszko et al. 2007). Remodeling of lung tissues during the process of granuloma formation requires restructuring of the extracellular matrix and cathepsins K, L, and S are among the strongest extracellular matrix degrading enzymes (Samokhin et al. 2011). According to Samokhin et al. (2011), lack of cathepsin activities alters or prevents the development of lung granulomas in a mouse model of Besnier-Boeck-Schaumann (BBS) disease, sarcoidosis. One of the newly discovered proteolytic enzymes is cathepsin V (Cath V). There have so far been no data about concentrations of Cath V in BALF in humans.

There are many reports about increased angiogenesis in patients with pulmonary BBS. Increased angiogenesis-inducing ability of activated alveolar macrophages has been found in bronchoalveolar specimens from patients with pulmonary sarcoidosis (Fireman et al. 2009). Endostatin, an anti-angiogenic peptide, is a novel inhibitor of distal lung epithelial cells and primary type II cells (Richter et al. 2009). This protein has not yet been investigated in BBS patients.

The clinical course of the BBS may be different; from spontaneous remission to severe and chronic form. New markers of sarcoidosis are highly desirable because they can improve the diagnosis and treatment. In our study we examined Cath V and endostatin in BALF of BBS patients. We correlated these proteins with IL-18, increased in bronchoalveolar lavage fluid (BALF) in active sarcoidosis (Kieszko et al. 2007).

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation of the World Medical Association and the protocol was approved by a local Ethics Committee. Written informed consent was obtained from all participants.

2.1 Subjects

The study group consisted of 22 BBS patients (Stage 2, with bilateral hilar lymphadenopathy and pulmonary infiltrations; F/M – 4/18, mean age 47 ± 9 years) consecutively recruited at the Department of Lung Diseases, the Medical University of Bialystok, Poland between 2007 and 2011. The diagnosis of BBS was based on clinical and pathological criteria (Statement on sarcoidosis 1999). The control group consisted of 20 healthy volunteers (F/M – 3/17 men; mean age 49 ± 7) without any acute or chronic inflammatory conditions. Both patients and healthy subjects underwent BALF and lung function tests including spirometry and diffusing capacity for carbon monoxide (DL_{CO}) (Standardization of Spirometry, 1994 Update; American Thoracic Society (ATS) 1995). Bronchoscopy and BALF in all patients was performed as part of a routine clinical workup. Bronchoalveolar lavage was made using fiberoptic bronchoscopy (Pentax FB 18 V; Pentax Corporation, Tokyo, Japan) under local anesthesia with lidocaine, following premedication with intramuscular atropine and hydroxyzine as a sedative. The bronchoscope was wedged in the right middle lobar bronchus and three 50 ml aliquots of sterile 0.9 % saline were gently instilled and recovered by suction. The recovered fluid was collected and stored on ice and processed within 1 h. Recovered fluid was sieved through a layer of sterile gauze and centrifuged at 800 rpm for 10 min at 4 °C. Supernatant was stored at −70 °C until use. BALF samples were analyzed for total and differential cell counts, Cath V, endostatin, and IL-18 levels detected by Elisa. These results were expressed

as cells $\times 10^5$/ml. The differential cell profile was made by counting at least 400 cells under a light microscope (magnification $\times$ 1,000). Another part of the cell suspension was incubated with phycoerythrin-labeled anti-CD4 antibody (Becton Dickinson, Mountain View, CA), and fluorescein isothiocyanate-labeled anti-CD8 antibody (Becton Dickinson, Mountain View, CA) for 20 min, washed twice, and resuspended for flow cytometry. The stained cells were analyzed on a flow cytometer (Becton Dickinson, Mountain View, CA). Lymphocytes were gated on forward and side scatter, and the percentages of positively stained cells were scored to determine the number of CD4 and CD8 cells.

2.2 Concentrations of Cathepsin V, Endostatin and IL-18 in BALF

Cathepsin V, endostatin, and IL-18 were analyzed in BALF with quantitative test kits and concentrations were determined by means of enzyme-linked immunosorbent assay (ELISA) method (R&D System, Minneapolis, MN). All specimens were assayed in duplicates. The minimum detectable levels of Cath V, endostatin, and IL-18 were 0.95 pg/ml, 0.001 ng/ml, and 10.5 pg/ml, respectively.

2.3 Statistical Analysis

Data distribution was checked with the Shapiro Wilk test. A *t*-test for independent or dependent data was used to compare respective groups and pairs. The Wilcoxon and Mann-Whitney U tests were used for the features inconsistent with the normal data distribution. Correlations were calculated by the Spearman rank test. Receiver-operating characteristics (ROC) curves were constructed to find the cut-off levels of Cath V, endostatin, and IL-18. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using Statistica 10.0 software (Stat Soft Inc., Tulsa, OK).

3 Results

There were no significant differences in age or gender between patients and healthy subjects. Pulmonary function analysis revealed that %VC and $\%DL_{CO}$ were significantly reduced in patients with BBS in comparison with the control group (%VC: 87.4 ± 17.1 *vs.* 98.1 ± 4.0, $p = 0.03$; $\%DL_{CO}$: 83.3 ± 30 *vs.* 92.5 ± 12.7, $p = 0.02$).

There was no difference in the fluid recovery rate of bronchoalveolar lavage (BAL) between the two investigated groups. Patients with BBS had a higher percentage of lymphocytes and a lower percentage of macrophages (%lymphocytes: 42.4 ± 21.0 *vs.* 18.0 ± 8.0, $p = 0.0002$; %macrophages: 55.5 ± 21.0 *vs.* 80.1 ± 17.0, $p = 0.004$). The percentage of CD4+ was higher in BALF of BBS patients than in healthy subjects (%CD4+: 46.6 ± 16.0 *vs.* 9.3 ± 0.4, $p = 0.009$). There was a similar percentage of CD8+ in BBS and healthy subjects (%CD8+: 17.8 ± 4.8 *vs.* 19.1 ± 5.0, $p = 0.231$).

The BALF levels of Cath V, endostatin, and IL-18 are shown in Fig. 1a–c. Cath V concentration was lower in BBS than in healthy subjects (16.03 ± 8.60 *vs.* 32.25 ± 21.90 pg/ml, $p = 0.004$). Both endostatin and IL-18 levels were higher in BBS than in control group (0.88 ± 0.30 *vs.* 0.29 ± 0.04 ng/ml, $p = 0.028$; 40.37 ± 31.60 *vs.* 14.61 ± 1.30 pg/ml, $p = 0.007$).

ROC curves for Cath V, endostatin, and IL-18 in BALF were applied to determine the cut-off values. Sensitivity and specificity of Cath V levels in the BBS patients relative to the healthy group were 90 % and 50 %, respectively, at a cut-off value of 28.08 pg/ml. Sensitivity and specificity of endostatin levels in the BBS patients relative to the healthy subjects were 71 % and 8 %, respectively, at a cut-off value of 0.39 ng/ml. Sensitivity and specificity of IL-18 levels in the BBS patients relative to the healthy subjects were 81 % and 28 %, respectively, at a cut-off value of 14.21 pg/ml. The areas under the curve for Cath V, endostatin, and IL-18 in BALF were 0.74, 0.84 and 0.79, respectively (Fig. 2).

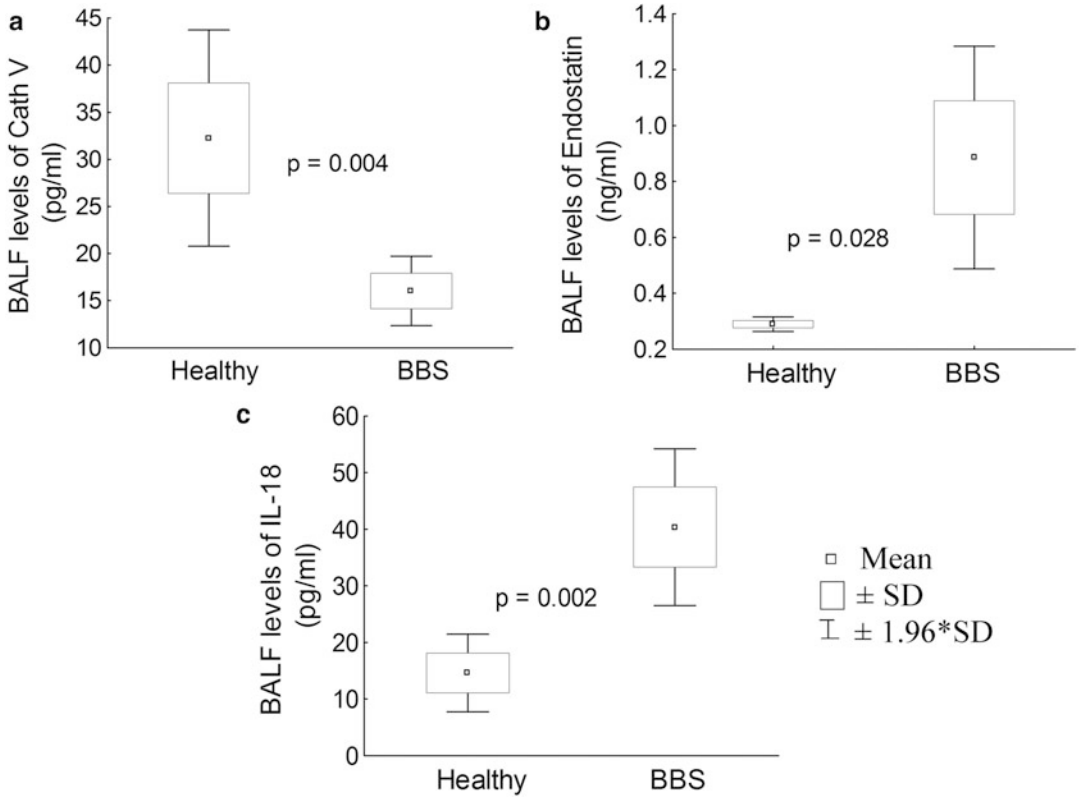

Fig. 1 **Decreased Cath V** (**a**), **increased endostatin** (**b**) **and IL-18** (**c**) in BALF of Besnier-Boeck-Schaumann disease (BBS) patients as compared with healthy volunteers

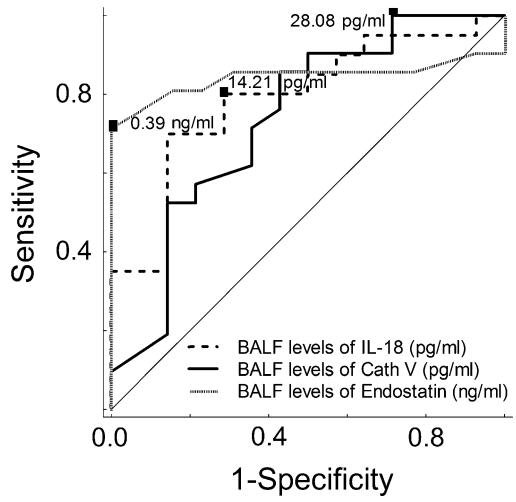

Fig. 2 **Receiver operating characteristic (ROC) curve** for Cath V, endostatin, and IL-18 in BALF differentiating BBS and healthy subjects (AUC 0.74, 0.84, and 0.79, respectively)

In the BBS group, a positive correlation was found between the BALF levels of endostatin and IL-18 ($r = 0.74$, $p < 0.001$) (Fig. 3). We observed a negative correlation between BALF levels of endostatin and $\%DL_{CO}$ in the BBS group (Fig. 4). Moreover, in the BBS group, BALF concentrations of endostatin correlated with following parameters: %lymphocytes ($r = 0.52$, $p = 0.019$), %macrophages ($r = 0.52$, $p = 0.018$) and CD4+/CD8+ ($r = 0.86$, $p = 0.013$). Cath V concentration negatively correlated with CD4+/CD8+ in BALF of BBS patients ($r = -0.83$, $p = 0.003$).

4 Discussion

In the present study we revealed that patients with BBS had a lower level of Cath V in BALF than healthy people. To our knowledge, this

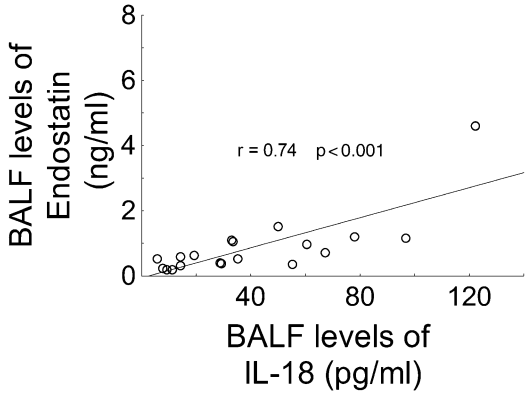

Fig. 3 Correlation between concentrations of endostatin, and IL-18 in BALF of Besnier-Boeck-Schaumann disease (BBS) patients

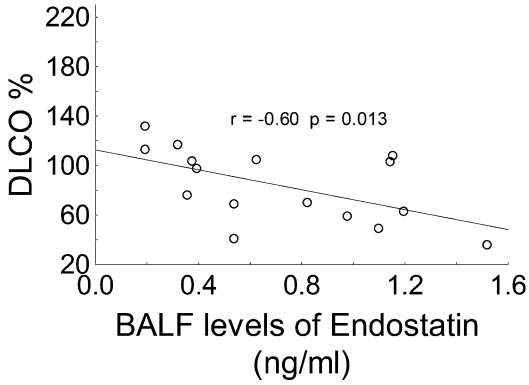

Fig. 4 Correlation between endostatin and DL_{CO} (diffusing capacity for carbon monoxide) in BALF of Besnier-Boeck-Schaumann disease (BBS) patients

study is the first concerning the cathepsin concentration in BALF of humans. Our study is consistent with the findings of Samokhin et al. (2011) in the mouse lungs. That study, which focused on the cathepsin K, L, and S, showed that lack of cathepsin activity prevents the development of lung granulomas. These cysteine proteases degrade major extracellular proteins (Shi et al. 1992) and are involved in immune responses (Hsing and Rudensky 2005; Honey and Rudensky 2003). Cathepsins L and S play a significant role in the antigen presentation and T cell selection (Honey and Rudensky 2003; Nakagawa et al. 1998), and the formation of granulomas has been linked to T cell activation (Gerke and Hunninghake 2008; Grunewald and Eklund 2007; Noor and Knox 2007). The role of Cath V in humans has not yet been clarified, but *in vitro* experiments have demonstrated that it has similar physiological properties to cathepsin S (Berdowska 2004). Possible explanation for the decreased Cath V level, in the present study, is proteolytic degradation. Pulmonary sarcoidosis is associated with chronic lung inflammation; thus, it is possible that pro-inflammatory protease may degrade Cath V and lead to its reduced level in BALF. We presume that a low level of Cath V in BBS patients is due to its being used up during the destruction of the extracellular matrix. This process is essential to the lung granuloma formation (Gerke and Hunninghake 2008; Grunewald and Eklund 2007; Noor and Knox 2007). A negative correlation between the BALF level of Cath V and CD4+/CD8+ is consistent with the hypothesis above outlined. Lymphocytic alveolitis with predominance of CD4+Th cells and macrophages is typical in sarcoidosis (Grunewald and Eklund 2007).

Several studies on cathepsins have demonstrated that these enzymes participate in signaling pathways leading to apoptosis (Turk et al. 2002). Apoptosis plays an important role in the resolution of granulomas (van Maarsseveen et al. 2009). Recently, it has been demonstrated that BBS lymphocytes CD4+ present resistance to apoptosis (Dubaniewicz et al. 2006). Petzmann et al. (2006) described decreased apoptosis of antigen-primed T cells in BALF of BBS patients. These findings are in accord with our study because BBS patients had lower levels of Cath V than healthy subjects.

Shi et al. (2003) described that cathepsins are involved in the controlled extracellular matrix degradation, enabling endothelial cells to penetrate the vascular basement membranes to form new vessels. Several investigators confirmed that in pulmonary BBS the angiogenic/angiostatic balance is also disturbed. Fireman et al. (2009) reported that VEGF in the alveolar space is lower in BBS than in healthy subjects. The authors also revealed that the level of VEGF in patients at Stage 3–4 of disease is significantly lower than that at Stage 1–2, indicating a less fibrotic parenchymal disorder in the latter.

In the present study, the level of endostatin was higher in the BBS than in control subjects. This may indicate the inhibition of angiogenesis in patients with sarcoidosis. Our results are similar to those reported by Richter et al. (2009). They confirmed that endostatin reduces migration and spreading of endothelial cells, and induces epithelial apoptosis. Moreover, macrophages are also known to secrete anti-angiogenic factors, such as endostatin/collagen XVIII (Kamboucher et al. 2011). We confirmed that report as endostatin correlated with the percentage of macrophages in BALF. Kamboucher et al. (2011) found a correlation between endostatin and lung function impairment. We also observed a negative correlation between the level of endostatin and DL_{CO}. The presence of endostatin within the lung in BBS may result in an alveolar epithelial repair. Another possible explanation for increased endostatin level is proteolytic degradation of proangiogenic stimulators. The relationship between endostatin and IL-18 in BALF indicates a potentially important clinical role of our observations.

Recent studies have reported that IL-18 is closely related to the pathogenesis of pulmonary sarcoidosis (Shigehara et al. 2001). IL-18 plays an important role in the induction of the Th1 response and it may be responsible for BBS progression and granuloma formation. Moreover, it plays a pivotal role in linking inflammatory immune responses and angiogenesis in pulmonary BBS (Amin et al. 2010).

In summary, our findings, although obtained in a small number of patients, show a significant relationship between the level of endostatin and DL_{CO} in BALF of BBS patients. Patients with the more severe impairment of DL_{CO} had a higher concentration of endostatin – an inhibitor of angiogenesis. Plausibly, an endostatin inhibitor may become a therapeutic option in BBS in the future.

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Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.

References

American Thoracic Society (1995) Single-breath carbon monoxide diffusing capacity (transfer factor). Recommendations for a standard technique-1995 update. Am J Respir Crit Care Med 152:2185–2198

Amin MA, Rabquer BJ, Mansfield PJ, Ruth JH, Marotte H, Haas CS, Reamer EN, Koch AE (2010) Interleukin 18 induces angiogenesis *in vitro* and *in vivo via* Src and Jnk kinases. Ann Rheum Dis 69:2204–2212

Berdowska I (2004) Cysteine proteases as disease markers. Clin Chim Acta 342:41–69

Dubaniewicz A, Trzonkowski P, Dubaniewicz-Wybieralska M, Dubaniewicz A, Singh M, Myśliwski A (2006) Comparative analysis of mycobacterial heat shock proteins-induced apoptosis of peripheral blood mononuclear cells in sarcoidosis and tuberculosis. J Clin Immunol 26:243–250

Fireman E, Gilburd D, Marmor S (2009) Angiogenic cytokines in induced sputum of patients with sarcoidosis. Respirology 14:117–123

Gerke AK, Hunninghake G (2008) The immunology of sarcoidosis. Clin Chest Med 29:379–390

Grunewald J, Eklund A (2007) Role of CD4+ T cells in sarcoidosis. Proc Am Thorac Soc 4:461–464

Honey K, Rudensky AY (2003) Lysosomal cysteine proteases regulate antigen presentation. Nat Rev Immunol 3:472–482

Hsing LC, Rudensky AY (2005) The lysosomal cysteine proteases in MHC class II antigen presentation. Immunol Rev 207:229–241

Kamboucher M, Pirici D, Uhl JF, Mogoanta L, Valeyre D, Bernaudin JF (2011) Lymphatic and blood microvasculature organisation in pulmonary sarcoid granulomas. Eur Respir J 37:835–840

Kieszko R, Krawczyk P, Jankowska O, Chocholska S, Król A, Milanowski J (2007) The clinical significance of interleukin 18 assessment in sarcoidosis patients. Respir Med 101:722–728

Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, Villadangos JA, Ploegh H, Peters C, Rudensky AY (1998) Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. Science 280:450–453

Noor A, Knox KS (2007) Immunopathogenesis of sarcoidosis. Clin Dermatol 25:250–258

Petzmann S, Maercker C, Markert E, Kern I, Osolnik K, Pohl W, Popper HH (2006) Enhanced proliferation and decreased apoptosis in lung lavage cells of sarcoidosis patients. Sarcoidosis Vasc Diffuse Lung Dis 23:190–200

Richter AG, McKeown S, Rathinam S, Harper L, Rajesh P, McAuley DF, Heljasvaara R, Thickett DR (2009) Soluble endostatin is a novel inhibitor of epithelial repair in idiopathic pulmonary fibrosis. Thorax 64:156–161

Samokhin AO, Gauthier JY, Percival MD, Brömme D (2011) Lack of cathepsin activities alter or prevent the development of lung granulomas in a mouse model of sarcoidosis. Respir Res 20:12–13

Shi GP, Munger JS, Meara JP, Rich DH, Chapman HA (1992) Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. J Biol Chem 267:7258–7262

Shi GP, Sukhova GK, Kuzuya M, Ye Q, Du J, Zhang Y, Pan JH, Lu ML, Cheng XW, Iguchi A, Perrey S, Lee AM, Chapman HA, Libby P (2003) Deficiency of the cysteine protease cathepsin S impairs microvessel growth. Circ Res 92:493–500

Shigehara K, Shijubo N, Ohmichi M, Takahashi R, Kon S, Okamura H, Kurimoto M, Hiraga Y, Tatsuno T, Abe S, Sato N (2001) IL-12 and IL-18 are increased and stimulate IFN-gamma production in sarcoid lungs. J Immunol 166:642–649

Standardization of Spirometry, 1994 Update. American Thoracic Society (1995) Am J Respir Crit Care Med 152:1107–1136

Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee (1999) Am J Respir Crit Care Med 160:736–755

Turk B, Stoka V, Rozman-Pungercar J, Cirman T, Droga-Mazovec G, Oresić K, Turk V (2002) Apoptotic pathways: involvement of lysosomal proteases. Biol Chem 383:1035–1044

van Maarsseveen TC, Vos W, van Diest PJ (2009) Giant cell formation in sarcoidosis: cell fusion or proliferation with non-division? Clin Exp Immunol 155:476–486