THERAPY FOR ALPHA-1 ANTITRYPSIN DEFICIENCY: THE EVIDENCE FOR EFFICACY

This symposium took place on 28th September 2015 as part of the European Respiratory Society International Congress 2015 in Amsterdam, Netherlands

Chairperson

Noel Gerard McElvaney[1]

Speakers

Emer Reeves,[2] David Parr,[3] Niels Seersholm,[4] Kenneth R. Chapman[5]

1. Department of Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland
2. Beaumont Hospital, Royal College of Surgeons in Ireland, Dublin, Ireland
3. University Hospitals Coventry and Warwickshire, Coventry, UK
4. Gentofte Hospital, Hellerup, Denmark
5. University of Toronto, Toronto, Ontario, Canada

Disclosure: David Parr has received honoraria for advising CSL Behring and Grifols (formerly Talecris), in addition to sponsorship from AstraZeneca and Boehringer Ingelheim. Emer Reeves and Niels Seersholm have no competing interests to disclose; however, both have received honoraria for this educational activity funded by CSL Behring. Kenneth R. Chapman has received compensation for consulting with AstraZeneca, Baxter, Boehringer Ingelheim, CSL Behring, GlaxoSmithKline, Grifols, Kamada, Novartis, Nycomed, Roche, and Telacris; has completed research funded by Amgen, AstraZeneca, Baxter, Boehringer Ingelheim, CSL Behring, Forest Labs, GlaxoSmithKline, Grifols, Novartis, Roche, and Takeda; and has participated in continuing medical education activities sponsored in whole or in part by AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Grifols, Merck Frosst, Novartis, Pfizer, and Takeda. He is participating in research funded by the Canadian Institutes of Health Research operating grant entitled Canadian Cohort Obstructive Lung Disease. He holds the GlaxoSmithKline–Canadian Institutes of Health Research Chair in Respiratory Health Care Delivery at the University Health Network, Toronto, Ontario, Canada.
Acknowledgements: Writing assistance was provided by Ms Rachel Stevens of ApotheCom.
Support: The publication of this article was funded by CSL Behring.
Citation: EMJ Respir. 2015;3[2]:55-62.

MEETING SUMMARY

Prof McElvaney opened the symposium with a brief overview of the disease history and available treatments to date for alpha-1 antitrypsin deficiency (AATD). He then introduced Dr Reeves, who gave a description of the physiological function of alpha-1 proteinase inhibitor (α1-PI), specifically its effect on neutrophil function in AATD. Dr Parr then discussed the limitations of using forced expiratory volume (FEV_1) to observe lung disease progression, and the development and use of measurements of lung density as an alternative. Dr Seersholm followed with a comprehensive overview of recent clinical studies demonstrating the efficacy of α1-PI augmentation therapy. Dr Chapman gave the final presentation that expanded on this by describing the findings of the randomised, placebo-controlled trial of augmentation therapy in α1-PI deficiency (RAPID) study. The meeting objectives were to present the current treatment landscape for AATD-associated emphysema and the role of α1-PI therapy within this.

Welcome and Introduction

Professor Noel Gerard McElvaney

AATD was first described in 1963.[1] Subsequent studies linked AATD to both familial emphysema[2,3] and liver disease,[4] and also demonstrated a relationship between α1-PI and neutrophil elastase.[5] The first α1-PI therapy, Prolastin®, received FDA approval in the USA in 1987 and received approval in European countries in 1989. The efficacy

endpoint of lung density for α1-PI augmentation therapy was first suggested in 1999 by a Danish/Dutch trial[6] that showed a trend toward the slowing of lung density decline in AATD patients receiving α1-PI augmentation therapy versus placebo. In part as a result of this finding, a statement was published by the American Thoracic Society (ATS) and European Respiratory Society (ERS) in 2003 regarding the standards for diagnosis of AATD.[7] In 2003, Zemaira®, the first second-generation α1-PI product, was granted FDA approval in the USA. Second-generation α1-PI products are highly purified (≥90% α1-PI), with lower infusion volumes and shorter infusion times than their first-generation counterparts.

In 2009, the use of computed tomography (CT) densitometry was encouraged further by the publication of the EXAcerbations and Computed Tomography scan as Lung End-points (EXACTLE) trial, which demonstrated a trend toward slowing of emphysema progression.[8] The RAPID study has since demonstrated the clinical efficacy of the second-generation α1-PI therapy Respreeza® in patients with AATD-associated emphysema.[9] Respreeza has now received EU market authorisation.

Anti-Inflammatory Properties of α1-PI Augmentation Therapy

Doctor Emer Reeves

α1-PI is an acute phase protein predominantly synthesised by hepatocytes. As the main inhibitor of various neutrophil-derived serum proteases, such as proteinase 3, cathepsin G, and neutrophil elastase, its key role is to maintain a protease/antiprotease balance in the airways.[10]

AATD is the only clearly identified genetic risk factor for chronic obstructive pulmonary disease (COPD). The most common allele variant, Z, is produced by a single amino acid substitution in the *SERPINA1* gene that leads to polymer formation and protein retention in the liver, thus reducing concentrations of circulating Z-AAT to approximately 10%.[10]

As the main source of the proteolytic burden, neutrophils are of great importance in the pathogenesis of AATD. α1-PI also regulates neutrophil function and plays a vital role in neutrophil adherence, chemotaxis, degranulation, apoptosis, and associated changes in neutrophil properties in AATD individuals. In addition, neutrophil adherence is activated by leukotriene B4 (LTB4) signalling via BLT-1 receptors.

Confocal microscopy has shown α1-PI bound to the outer membrane of neutrophils,[11] with significantly higher levels associated with the outer membrane of MM (normal control) neutrophils versus ZZ neutrophils, and significantly increased levels associated with the outer membrane of neutrophils in an AATD patient who had received a liver transplant.[11]

The Effect of α1-PI on Neutrophil Adherence, Chemotaxis, and Degranulation

α1-PI has been shown to reduce neutrophil adherence and also exhibit an inhibitory effect on LTB4 response in MM and ZZ genotype neutrophils.[12] Furthermore, it was shown that levels of α1-PI associated with the outer membrane of ZZ neutrophils were lower, but significantly increased following therapy.[12,13]

Increased neutrophil elastase activity was also observed on neutrophils isolated from ZZ individuals with or without COPD. It is possible that neutrophil elastase could trigger the synthesis of LTB4, as these individuals also exhibited higher plasma LTB4 levels.[12] However, augmentation therapy significantly decreased neutrophil elastase activity and lowered plasma LTB4 concentrations.[12]

An increase in neutrophil chemotaxis was observed in patients with AATD who underwent bronchoalveolar lavage.[14] Tumour necrosis factor alpha (TNFα), a factor associated with COPD which causes degranulation of neutrophils,[15,16] has also been found in significantly higher levels on the membranes of ZZ neutrophils compared with those of MM neutrophils.[17] AATD patients receiving α1-PI augmentation therapy exhibited significantly lower levels of TNFα.[17]

Q&A session

Do you think the effects you are seeing are dose-related?

Dr Reeves: A dose effect was found using *in vitro* experiments, where doses of up to 27.5 µmol/L effectively inhibited neutrophil response to IL-8, TNFα, and LTB4. It is difficult to confirm if these effects were inhibited altogether. Certain concentrations of IL-8 will overcome 27.5 µmol/L α1-PI, which is important to allow the neutrophil to transmigrate and kill bacteria.

The Relevance of CT Lung Density for Assessing Emphysema in AATD

Doctor David Parr

FEV_1 has been the gold standard surrogate measure for emphysema, although there are various limitations to this method. A study conducted with several hundred patients over several years found very little FEV_1 decline in patients with both mild and severe disease.[18] Despite the large number of patients, the measure showed broad variability and was not a sensitive measure of disease progression. In order to achieve sufficient power, 494 subjects per arm would be required to detect a treatment effect over 3 years if FEV_1 was used as an outcome measure.[19]

So-called 'modern definitions' of emphysema have come to recognise dilatation of lung air spaces and destruction of the walls,[20] along with the importance of excluding conditions that have obvious fibrosis, although this can be difficult to distinguish from emphysema.[21] More recently, the principles of design-based stereology recognise the importance of measuring lung dimensions in inflation.[22] Panlobular and centrilobular emphysema, which present the same basic structure seen in histopathological images of the lungs, can be distinguished using CT scans. In CT imaging, each pixel in the images relates to a volume of lung tissue, hence the term voxel is used, and each voxel has a value which is a measure of the density of the corresponding volume of lung tissue. Voxel distribution histograms can therefore be used to generate densitometric indices, while the percentile point method for monitoring over time has been used more recently due to its superior sensitivity.[23] These methods are of great importance in quantifying emphysema and may also help to inform patients about the extent and progress of their disease. Emphysema distribution can also influence where treatment effects are seen. For example, Prolastin-treated patients showed the greatest reduction in loss of lung mass in the basal region of the lung when compared with the placebo group, although this was not statistically significant.[23]

The changes in lung size and dimensions that occur when breathing may affect precise measurements of lung volume. Assuming that lung mass is preserved whilst breathing, a linear volume–density relationship can be shown and the density measurements of CT scans can be adjusted according to changes in lung volume.[24] If volume is standardised (constant) then we can calculate the density change, which should indicate a change in lung mass.[24]

The Alpha-1 Detection and Programme for Treatment (ADAPT UK registry) programme measured changes in lung volume and compared this with changes in lung density over a 2-year period. A linear relationship was found between the two, the results of which indicated a loss of lung mass in the patients[25] – a relationship also demonstrated by the EXACTLE trial data, which show a reduction in lung mass when using Prolastin.[8]

As CT directly correlates with the histopathological gold standard for emphysema assessment when scanning at full inspiration, as well as with lung function, we now know that CT is a clinically relevant outcome measure. CT lung density reduction has proven to be linked to lung mass reduction and a deterioration in clinical measures, even though these studies are not powered to demonstrate a treatment effect using physiological outcomes.

Q&A session

One of the concerns with CT has been radiation dose. Would you like to comment on that?

Dr Parr: The protocols developed have reduced the radiation dose further while maintaining good reproducibility, even at low doses. However, errors in densitometric analysis may arise in patients with a large body mass index when very low dose protocols are used. With increasing use of CT in clinical practice, there has been concern regarding inappropriate use of imaging for diagnosis and the associated radiation risks, but the radiation dose of densitometric scans is significantly lower than that of diagnostic imaging.

Where do you see the advances in CT over the next number of years in this area?

It is likely that more studies will combine inspiratory and expiratory images, which will allow mapping and registering of different parts of the image together, even when there is movement. Further improvements in magnetic resonance imaging may well displace CT as the gold standard measure of emphysema.

Efficacy of α1-PI Augmentation Therapy – a Review of Evidence

Doctor Niels Seersholm

It was first shown in 1987 that weekly doses of α1-PI augmentation therapy can significantly increase α1-PI plasma levels.[26] Despite the inability to do large-scale, long-term studies based on lung function, the α1-PI treatment was approved in several countries following this study.[25-28]

A decade later, a Danish/German registry study followed up on these findings by observing FEV_1 decline over time in patients who were treated with α1-PI augmentation therapy.[29] German patients who were treated with augmentation therapy exhibited a significantly slower rate in FEV_1 decline compared with the non-treated Danish patients ($p<0.04$).[29]

These findings were subsequently echoed by a larger American registry study of 1,129 patients who had never, always, or partly received augmentation therapy.[18] THE ATS/ERS guidelines published as a result of the aforementioned studies advised that augmentation therapy should be used for patients with FEV_1 between 30% and 65% of that predicted.[7]

A year later, a placebo-controlled, parallel, double-blind Danish/Dutch study of α1-PI versus placebo[6] reported that pulmonary function tests showed a faster rate of FEV_1 decline in the active treatment group compared with placebo. However, CT scan results did show a slower change in lung density and a trend toward benefit in the active treatment group ($p<0.07$).[6] It was clear from this study that power calculations based on annual decline in FEV_1 are not feasible due to the large number of patients required for this method.[6] CT lung density scans may better facilitate future randomised controlled trials as lower numbers of patients are needed to power the analyses.[6]

The role of CT densitometry was further investigated a decade later in the EXACTLE trial.[8] Similarly to the Danish/Dutch study, the trial revealed a reduced rate of lung density loss in the treatment arm versus placebo ($p<0.07$). No effect on lung function, quality of life, or mortality was observed, indicating again that CT densitometry is a more sensitive outcome measure than physiology and health status.[8]

The RAPID Trial: Evidence for the Efficacy of α1-PI Augmentation Therapy

Doctor Kenneth R. Chapman

For the RAPID study, 180 patients were randomised in a double-blind fashion and treated with either 60 mg/kg per week of a highly purified α1-PI (Respreeza) or placebo over the course of 2 years. Patients were well-matched for baseline characteristics and CT scans were performed at baseline, 3, 12, 21, and 24 months. A higher withdrawal rate was seen in the placebo group due to slightly higher rates in death, adverse events, and withdrawn consent.[9]

The RAPID trial is the first adequately powered, randomised clinical trial that has been able to demonstrate statistically significant efficacy of α1-PI in slowing emphysema progression. The main outcome of the RAPID trial was that α1-PI therapy slowed the progression of emphysema as measured by CT lung density, which is a more sensitive measure of disease progression in AATD-associated emphysema than conventional lung function parameters. Over a 2-year period, the difference in annual rate of decline in adjusted CT scan lung density was 0.74 g/L in favour of α1-PI when measured at full inspiration (total lung capacity [TLC]; Figure 1). The change in lung density was significantly different between treated and placebo groups ($p=0.033$, two-sided test).[9]

The reduced decline in lung density, i.e. slower rate of lung tissue destruction, under treatment with Respreeza is expected to extend the time towards terminal respiratory function (i.e. death, lung transplantation, or severe respiratory dysfunction). Results from the RAPID trial were extrapolated over time to calculate the time until a terminal lung density of approximately 20 g/L would be achieved. This limit of 20 g/L was the average level of lung density of five RAPID trial patients who exited the study due to death, lung transplantation, or severe respiratory dysfunction.[9] Results from the RAPID trial suggest that the time to predicted terminal respiratory function is 12 years in placebo subjects, but is increased by approximately 6–18 years in Respreeza-treated patients (Figure 2).[9]

The RAPID trial was not large or long enough to assess differences in traditional lung function outcomes. Although no statistically significant differences between the augmented and non-augmented groups were observed in terms of FEV_1 or diffusing capacity of lung for carbon monoxide

(DLCO), statistically significant cross-sectional correlations were found between CT scan density and DLCO and FEV_1 as a percentage of predicted and St George's Respiratory Questionnaire activity scores both at the beginning and the end of the study, respectively.[9]

Patients who completed the 2-year treatment and observation period of the RAPID trial outside the USA were invited to participate in the RAPID Extension trial for an additional 2 years. All participants in the RAPID Extension trial received Respreeza at 60 mg/kg body weight per week, including those who were on placebo during the RAPID trial. The results presented below (Figure 3) are based upon the 97 patients who, as of December 2013, had completed both the RAPID and RAPID Extension trial. Patients in the Early-Start cohort maintained a therapeutic effect across 4 years of treatment, whereas patients in the Delayed-Start cohort showed a clear inflection in the slope of decline, i.e. a slower rate of lung density decline after switching to Respreeza. Initial lung density loss in the Delayed-Start cohort during placebo treatment from Months 0–24 was not regained. The results support the conclusion of a disease-modifying effect of Respreeza.

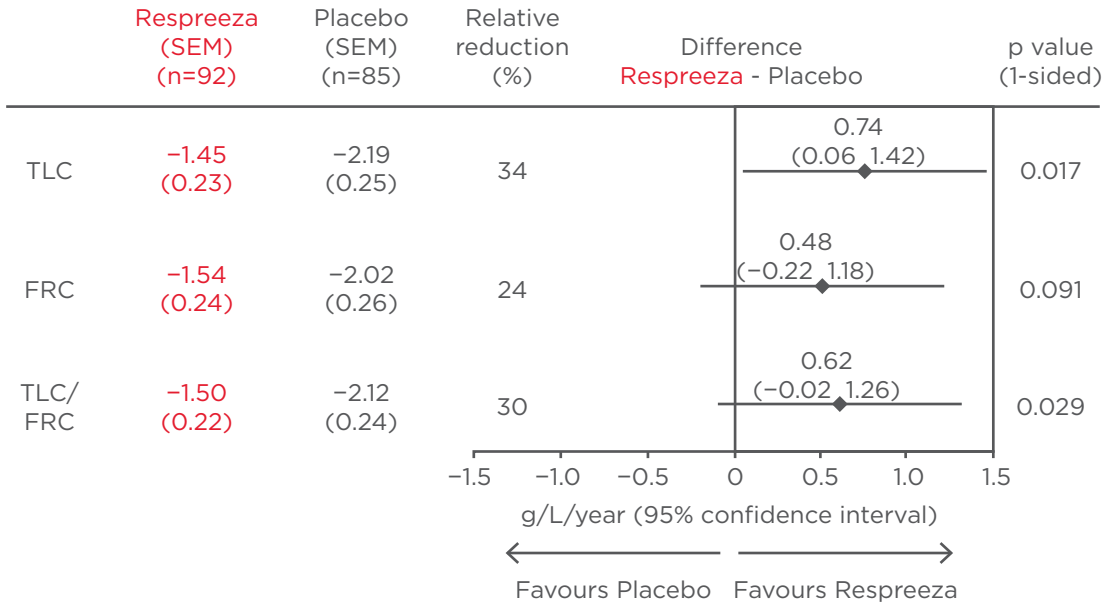

Figure 1: Primary outcome measure in the RAPID trial: difference in annual rate of decline of adjusted P15 (intention-to-treat population, physiologic adjustment).[9]

FRC: functional residual capacity; SEM: standard error of the mean; TLC: total lung capacity.

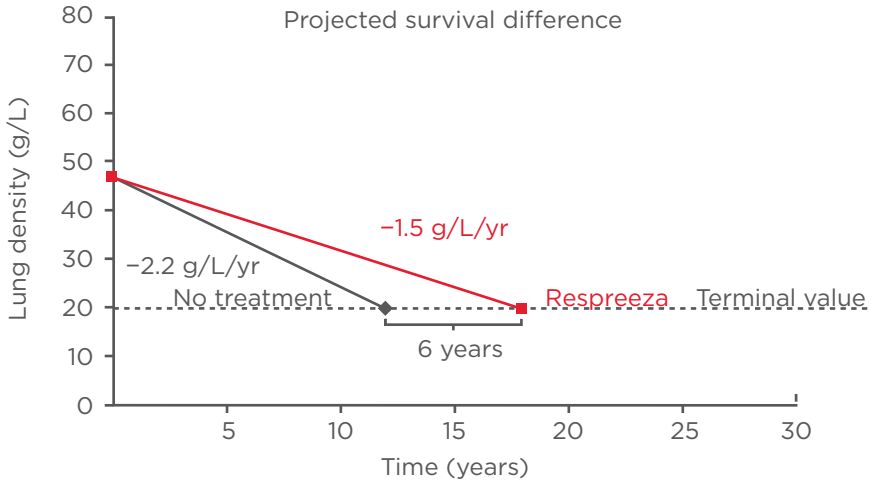

Figure 2: Potential life extension projected using RAPID study data.[9]

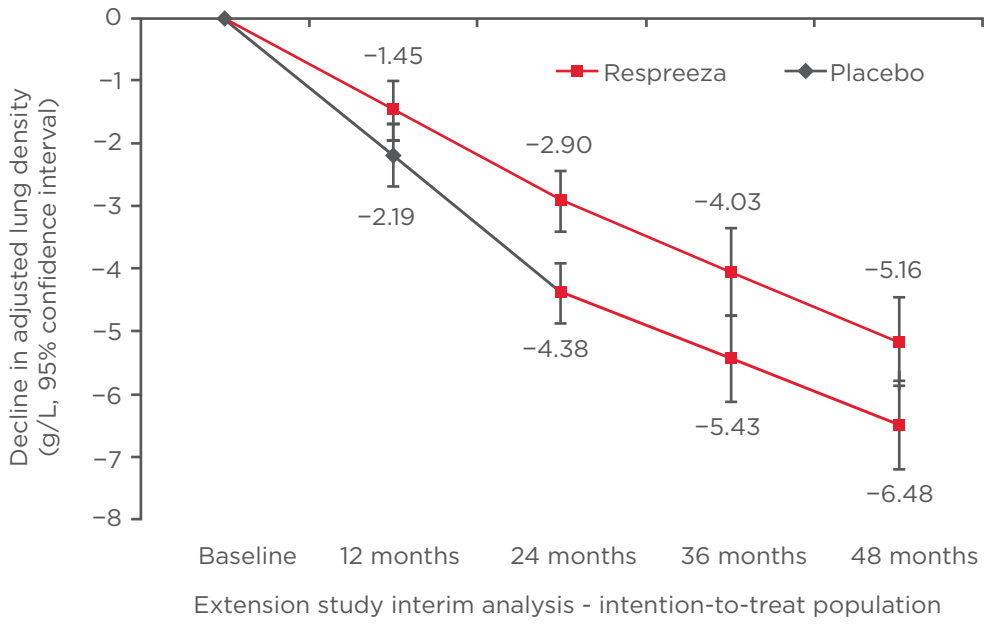

Figure 3: Decline in adjusted lung density over time (change from baseline).[9]

When considering whether further trials are required, it is not only important to consider the reproducibility of available data, but also the time required for patient recruitment and for the study to be carried out. For example, patient recruitment for the RAPID study took 7 years, along with the 2 years taken to conduct the trial. These results are very consistent with both the Danish/Dutch[8] and EXACTLE[6] trials in terms of decline in lung density (P15 decline TLC, g/L per year) as measured by CT scanning.[9]

Q&A session

When would you start augmentation therapy in a patient with AATD?

Dr Chapman stated that traditional measures of lung function are used as guides and that he would not wait for a further loss of FEV_1 to initiate therapy if the patient's FEV_1 at the time of diagnosis was below the predicted value. Dr Chapman then wondered whether a CT scan should also be performed, and if therapy might be initiated if emphysema were present radiologically while FEV_1 was relatively normal. Dr Chapman's viewpoint was that the presence of emphysema on a CT scan suggests that the patient has suffered from the loss of lung parenchyma, and that he would want to initiate therapy if coverage could be obtained.

Dr Seersholm then expressed his disapproval of the rule that the FEV_1 should be between 30% and 65% of that predicted, stating that augmentation therapy should be initiated when deterioration/holes in the lungs are shown by CT scan. He then stated the importance of defining the emphysema from the scan.

How do you think considerations of reimbursement influence the decision to initiate therapy?

Dr Chapman commented that the costs of augmentation therapy are similar to those of blood products for haemophilia treatment, but due to the immediate danger surrounding these bleeding events, the need for these products seems more apparent. The loss of lung function resulting in death or transplantation is so gradual that the need for therapy can be underestimated.

In the data shown by Dr Chapman, the higher values in the nadir level and α1-PI concentration seem to be matched with a better response. What are your thoughts on changing the current recommended dose of 60 mg/kg per week?

Dr Chapman recognised that an increase in dose will increase the cost, although this could be offset if a better result is seen. A dose of 120 mg/kg per week is currently under study. The investigation of other delivery schemes or schedules may be necessary, such as self-administration or more frequent and smaller doses to achieve the same serum level. However, Dr Chapman stated that more research is required before a decision is made on this.

How close did you get to a normal non-alpha rate of CT scan density decline in the RAPID trial?

Dr Parr stated that in future studies there will be a need to individualise and monitor the doses for patients. He mentioned that CT densitometry may not be the best method for this purpose, although using a biomarker may prove difficult in practice.

How do exacerbations and infections affect CT lung density measurements?

Dr Parr commented that most of the studies have a strict protocol with a window between any event and imaging, and as a consequence most of the exacerbations would not have been close to the timing of the CT scan. However, he stated that other factors have been known to affect CT lung density in observational studies, i.e. the development of pulmonary oedema brought on by a cardiac event or the development of pneumonia. Dr Parr then stressed the importance of contextualising any diagnostic test with the clinical situation and patient's medical history in order to avoid errors. This principle should be adopted when using CT as a tool to measure rather than diagnose.

REFERENCES

1. Laurell CB, Eriksson S. The electrophoretic α1-globulin pattern of serum in α1-antitrypsin deficiency. 1963. COPD. 2013;10 Suppl 1:3-8.

2. Eriksson S. Pulmonary emphysema and alpha1-antitrypsin deficiency. Acta Med Scand. 1964;175:197-205.

3. Ganrot PO et al. Obstructive lung disease and trypsin inhibitors in alpha-1-antitrypsin deficiency. Scand J Clin Lab Invest. 1967;19(3):205-8.

4. Sharp HL et al. Cirrhosis associated with alpha-1-antitrypsin deficiency: a previously unrecognized inherited disorder. J Lab Clin Med. 1969;73(6): 934-9.

5. Turino GM et al. Serum elastase inhibitor deficiency and alpha 1-antitrypsin deficiency in patients with obstructive emphysema. Science. 1969;165(3894):709-11.

6. Dirksen A et al. A randomized clinical trial of alpha(1)-antitrypsin augmentation therapy. Am J Respir Crit Care Med. 1999;160(5 Pt 1):1468-72.

7. American Thoracic Society, European Respiratory Society. American Thoracic Society/European Respiratory Society statement: standards for the diagnosis and management of individuals with alpha-1 antitrypsin deficiency. Am J Respir Crit Care Med. 2003;168(7):818-900.

8. Dirksen A et al. Exploring the role of CT densitometry: a randomised study of augmentation therapy in alpha1-antitrypsin deficiency. Eur Respir J. 2009;33(6):1345-53.

9. Chapman KR et al; RAPID Trial Study Group. Intravenous augmentation treatment and lung density in severe α1 antitrypsin deficiency (RAPID): a randomised, double-blind, placebo-controlled trial. Lancet. 2015;386(9991): 360-8.

10. McCarthy C et al. The role and importance of glycosylation of acute phase proteins with focus on alpha-1 antitrypsin in acute and chronic inflammatory conditions. J Proteome Res. 2014;13(7):3131-43.

11. Bergin DA et al. α-1 Antitrypsin regulates human neutrophil chemotaxis induced by soluble immune complexes and IL-8. J Clin Invest. 2010;120(12): 4236-50.

12. O'Dwyer CA et al. The BLT1 Inhibitory Function of α-1 Antitrypsin Augmentation Therapy Disrupts Leukotriene B4 Neutrophil Signaling. J Immunol. 2015; 195(8):3628-41.

13. Hurley K et al. Alpha-1 antitrypsin augmentation therapy corrects accelerated neutrophil apoptosis in deficient individuals. J Immunol. 2014; 193(8):3978-91.

14. Hubbard RC et al. Neutrophil accumulation in the lung in alpha 1-antitrypsin deficiency. Spontaneous release of leukotriene B4 by alveolar macrophages. J Clin Invest. 1991;88(3): 891-7.

15. Schols AM et al. Evidence for a relation between metabolic derangements and increased levels of inflammatory mediators in a subgroup of patients with chronic obstructive pulmonary disease. Thorax. 1996;51(8):819-24.

16. von Haehling S et al. Elevated TNFalpha production in whole blood in patients with severe COPD: the potential link to disease severity. Wien Klin Wochenschr. 2009;121(9-10):303-8.

17. Bergin DA et al. The circulating proteinase inhibitor α-1 antitrypsin regulates neutrophil degranulation and autoimmunity. Sci Transl Med. 2014;6(217):217ra1.

18. Survival and FEV1 decline in individuals with severe deficiency of alpha1-antitrypsin. The Alpha-1-Antitrypsin Deficiency Registry Study Group. Am J Respir Crit Care Med. 1998;158(1):49-59.

19. Schluchter MD et al. Feasibility of a clinical trial of augmentation therapy for alpha(1)-antitrypsin deficiency. The Alpha 1-Antitrypsin Deficiency Registry Study Group. Am J Respir Crit Care Med. 2000;161(3 Pt 1):796-801.

20. Terminology, Definitions, and Classification of Chronic Pulmonary Emphysema and Related Conditions: A report of the conclusions of a CIBA guest symposium. Thorax. 1959;14(4):286-99.

21. The definition of emphysema. Report of a National Heart, Lung, and Blood Institute, Division of Lung Diseases workshop. Am Rev Respir Dis. 1985;132(1):182-5.

22. Hsia CC et al; ATS/ERS Joint Task Force on Quantitative Assessment of Lung Structure. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. Am J Respir Crit Care Med. 2010;181(4):394-418.

23. Parr DG et al. Exploring the optimum approach to the use of CT densitometry in a randomised placebo-controlled study of augmentation therapy in alpha 1-antitrypsin deficiency. Respir Res. 2009;10:75.

24. Stoel BC et al. Volume correction in computed tomography densitometry for follow-up studies on pulmonary emphysema. Proc Am Thorac Soc. 2008;5(9):919-24.

25. Parr DG et al. Detection of emphysema progression in alpha 1-antitrypsin deficiency using CT densitometry; methodological advances. Respir Res. 2008;9:21.

26. Wewers MD et al. Replacement therapy for alpha 1-antitrypsin deficiency associated with emphysema. N Engl J Med. 1987;316(17):1055-62.

27. Galbán CJ et al. Computed

tomography-based biomarker provides unique signature for diagnosis of COPD phenotypes and disease progression. Nat Med. 2012;18(11):1711-5.

28. Parr DG et al. Validation of computed tomographic lung densitometry for monitoring emphysema in alpha1-antitrypsin deficiency. Thorax. 2006;61 (6):485-90.

29. Seersholm N et al. Does alpha1-antitrypsin augmentation therapy slow the annual decline in FEV1 in patients with severe hereditary alpha1-antitrypsin deficiency? Wissenschaftliche Arbeitsgemeinschaft zur Therapie von Lungenerkrankungen (WATL) alpha1-AT study group. Eur Respir J. 1997;10(10): 2260-3.

Click here to view the online CME activity on 'Clinical Evidence for Augmentation Therapy in Patients with Emphysema due to Alpha-1 Antitrypsin Deficiency (AATD)', available on Medscape.