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The commutability of enzyme linked immunosorbent assays for the quantification of serum eosinophil-derived neurotoxin (EDN)

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Abstract

Eosinophil-derived neurotoxin (EDN) is a surrogate biomarker of eosinophil activation and has considerable potential as a precision medicine biomarker in diseases where eosinophils may play a causative role. Clinical data for EDN have been generated using different quantitative immunoassays, but comparisons between these individual data sets are challenging as no internationally recognised EDN standards or orthogonal methods exist. In this study we aimed to compare commercial EDN assays from ALPCO, MBL, LSBio and CUSABIO for sample commutability. Firstly, we analytically validated the ALPCO enzyme

linked immunosorbent assay (ELISA) and demonstrated appropriate analytical characteristics, including an intra/inter-assay precision coefficient-of-variation of between 1.9-6.8%. EDN purified from blood proved to be a good quality control material, whereas recombinant EDN, expressed in *E.coli*, did not react in the ALPCO immunoassay. Using healthy and asthma patient serum samples we confirmed that the ALPCO assay correlated well with the MBL assay, with a coefficient of determination (R^2) of 0.92. However, the results from LSBio and CUSABIO assays were not commutable to the other assays.

Keywords: eosinophil-derived neurotoxin, EDN, ELISA, validation

1. Introduction

Eosinophil-derived neurotoxin is a small protein composed of a 134 amino acid polypeptide chain and a 27 residue N-terminus hydrophobic signal sequence, with a molecular weight of 18.4 kDa (Uniprot P10153). It contains 5 putative glycosylation sites (Cristina et al., 2015) that are likely to be important for its activity (Ye et al., 2006). Gleich (1986) confirmed that the native protein is glycosylated, increasing its molecular weight to ~20.1 kDa. A member of the ribonuclease A superfamily of proteins, EDN may play an important role in innate immunity, having been shown to have activity against single stranded viruses such as respiratory syncytial virus (Domachowske et al., 1997 & 1998). EDN is predominantly expressed by eosinophils, where it is stored within intracellular compartments known as granules. EDN was first purified to homogeneity from patients with hypereosinophilic syndrome and eosinophilic gastroenteritis (Durack et al., 1981), the gene was cloned a number of years later (Rosenberg et al., 1989) and the crystal structure subsequently solved for recombinant EDN (Mosimann et al., 1996).

Initially, EDN was measured by means of a crude bioassay using a principle called the Gordon phenomenon (Gordon, 1932). The intrinsic properties of EDN cause specific Purkinje cell degeneration when injected intracerebrally into rabbits. This assay was eventually superseded by immunohistochemical and radioimmunoassays (RIA) for both the detection and quantification of EDN (Molina et al., 1988; Reimert et al., 1991). However, RIA assays are time consuming and require the use of radioactive tracers. The development of an enzyme linked immunosorbent assay (ELISA) has made the quantitative measurement of EDN more accessible to researchers (Morioka et al., 2000). Access to EDN ELISAs has been aided by the availability of commercial, ready to use, kits that are suitable for most 96 well plate readers.

Using ELISA based technology, EDN has now been quantified in a number of different sample matrices including: plasma, serum, urine and sputum (Lee et al., 2019; Cottin et al., 1998; Panzer et al., 2003). There are constitutively low levels in the serum of healthy human individuals (Rutten et al., 2021). However, activated eosinophils can degranulate, releasing EDN into the surrounding tissue (Carr et al., 2016). Elevated levels of EDN have been found to correlate with disease severity in asthma (Lee et al., 2019). Additionally, blood levels have been shown to decrease in asthma after treatment with the inhaled corticosteroid budesonide and the anti-interleukin-5 receptor monoclonal antibody benralizumab, which depletes eosinophil numbers (Pedersen et al., 1996; Pham et al., 2016), demonstrating the value of this biomarker for monitoring the efficacy of anti-inflammatory therapeutics. EDN has also been proposed as a biomarker of lower airway disease, to follow the progression of patients with SARS-CoV-2 infections (Dosanjh, 2020).

In order to fully evaluate the potential of EDN as a biomarker we need to be able to compare and contextualise data obtained from different clinical studies using different assay methods. Commutability, the mathematical relationship between the results from different assays, is typically performed using certified reference materials and orthogonal methods (Vesper et al., 2007), which do not yet exist for EDN quantification. However, commutability can also be derived by using control materials and clinical samples from healthy and diseased subjects. In this study we validate the performance of the ALPCO EDN ELISA, selected because of our previous experience with the assay, and determined its commutability with other commercially available assays using serum samples, as well as assess the potential of native and recombinant EDN control proteins as future reference materials.

2. Materials and methods

2.1 Subjects and sample collection

Evaluation of the commutability of the EDN immunoassays was carried out using human serum samples obtained from 14 healthy subjects and 25 asthma patients recruited from Medicines Evaluation Unit, Manchester University NHS Foundation Trust (MFT), UK. Written informed consent was obtained from all participants (research ethics committee REC reference 10/H1016/25 approved by NRES Committee North West, Preston, UK) and the study was conducted in accordance with the UK Human Tissue Act (2004) and the 1964 Declaration of Helsinki.

2.2 EDN protein standards

Endogenous human EDN protein, purified from buffy coat leukocytes from volunteers, was obtained from commercial suppliers (Diagnostics Development, Sweden; 16-15-050414 Athens Research & Technology, US). Assessment of alternative reference materials was performed using recombinant human GST-EDN₁₋₁₆₁ protein (H00006036-P01, Novusbio, UK) produced in an *in-vitro* wheat germ expression system and recombinant 2His₆-SUMO-EDN₂₈₋₁₆₁ (MBS2097579, MyBiosource, US) expressed in *E.coli*.

2.3. SDS-polyacrylamide gel electrophoresis

EDN protein was solubilized in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulphate, 10% glycerol, 0.001% bromophenol blue and 1% 2-mercaptoethanol then boiled for 5 minutes. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 4-20% Tris-glycine gel system from Novex (Laemmli et al., 1970). Molecular weight markers were obtained from BioRAD. Protein bands were revealed by staining the gel with Coomassie brilliant blue G-250.

2.4 Analytical size exclusion chromatography (SEC)

10 ug of EDN protein was applied to a 3.2 x 300 cm Superdex 75 column (GE Healthcare) equilibrated with 0.02 M phosphate buffer (pH 7.2) containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 0.5 mL/min. The sample eluent absorbance was continuously monitored at 280 and 260 nm.

2.5 ALPCO EDN ELISA

EDN in serum was measured with a commercial ELISA (30-EDNHU-E01, ALPCO, US) according to the supplier's instructions. The microtiter plate, coated with capture anti-EDN monoclonal antibody, was washed 5 times with 250 µL of wash buffer in each well. Lyophilized calibration curve standards and controls (containing native/endogenous protein) were reconstituted in 500 µL ultrapure water. 100 µL of the calibration curve standards (0.25-16 ng/mL), controls (C1:[0.55 ng/mL] and C2:[2.63 ng/mL]) and samples (diluted 20-fold in assay buffer, giving an upper analytical measuring range of 320 ng/mL) were added in duplicate to the plate wells. The plate was covered tightly and incubated for 1 hour at room temperature (18-26°C) on a plate shaker. The contents of each well were then discarded and

the plate washed as before. Residual buffer was removed and 100 μ L of the detection anti-EDN peroxidase conjugated rabbit polyclonal antibody was added to each well and incubated for 1 hour. 100 μ L substrate (tetramethylbenzidine) was added into each well and the plate was incubated for 15 minutes, at room temperature, in the dark. 100 μ L acidic stop solution was added and the plate was read on a SpectraMax reader (Molecular Devices, US) at 450 nm against 620 nm as a reference. The EDN concentration of unknown samples were calculated from interpolation of the standard curve via a 4-parameter logistic fit using Softmax Pro GxP v5.0 software (Molecular Devices, US).

The ALPCO ELISA dilutional verification was assessed using four serum samples that were 2-fold serially diluted with assay buffer; acceptable recovery was $\pm$ 20% of the target concentration. The lower limit of quantification (LLOQ) was assessed using five levels of serum samples, assayed 25 times each over a period of 5 days. Intra-assay precision was determined by measuring 20 replicates of two EDN levels of serum controls within the same analytical run; inter-run precision was determined using two EDN levels of serum controls analysed once per day over a period of 10 days. Accuracy was determined using the ELISA kit controls, each measured 24 times. The results were evaluated against the target ranges provided within the Manufacturer's package insert.

2.6 EDN ELISA commutability

The commutability study assessed four different commercial Research Use Only EDN immunoassays: 1) EDN ELISA (ALPCO, US), 2) EDN ELISA Kit (7630, MBL International Corporation, US), 3) Human RNASE2 ELISA Kit (CSB-E17923h, CUSABIO Technology, US) and 4) Human RNASE2/EDN Sandwich ELISA Kit (LS-F12507, LifeSpan Biosciences (LSBio), US). The immunoassays were performed according to each manufacturer's protocol and read on a SpectraMax plate reader. A total of 39 individual serum samples were selected for measurement starting at an EDN level of 6.0 ng/mL and reaching 106.6 ng/mL, based on ALPCO quantification. Duplicate serum samples were analysed in a single assay plate for each ELISA.

3 Results

3.1 Performance of the ALPCO ELISA

The EDN ALPCO assay is a quantitative immunoassay that utilises a sandwich format with a monoclonal capture antibody and a polyclonal peroxidase-linked detection antibody.

The assay is described by the manufacturers to be appropriate for measuring EDN in urine, plasma and serum samples. In our laboratory, performing the ELISA using serum samples did not provide any challenges and, with a total run time of <2.5 hrs, it was possible to perform two runs per day. We determined the performance characteristics of the assay using a validation assessment in line with FDA and EMA bioanalytical guidelines. The assay has an analytical measuring range of 0-16 ng/mL but our dilutional linearity assessment allowed samples up to 40960 ng/mL to be quantitated. The manufacturer's package insert provides stool sample intra-run and inter-run precision coefficient-of-variation (CV) of 7.2-8.4% and 6.8-12.4%, respectively. With serum samples we found the intra-run and inter-run precision CVs to be lower across all quality control samples tested at 1.9-5.8% and 5.2-6.8%, respectively (Table 1).

Kit Calibrators	0.25, 1, 4, 16 ng/mL		
Analytical Measuring Range	0-16.0 ng/mL		
Dilution Verification	x64		
Lower Limit of Quantitation	10.0 ng/mL		0.25 ng/mL x 1:40 working dilution
Upper Limit of Quantitation	40960 ng/mL		x64 sample dilution x 1:40 working dilution
Intra-run Precision	QC1	62.7 ng/mL	1.9 %CV
	QC2	185.8 ng/mL	5.8 %CV
Inter-run Precision	QC3	60.8 ng/mL	5.2 %CV
	QC4	207.1 ng/mL	6.8 %CV
Accuracy	KC1	0.58 ng/mL	8.3 %CV
	KC2	3.26 ng/mL	6.1 %CV

Table 1. Analytical validation of ALPCO ELISA. QC, quality control; KC, kit control; CV, coefficient of variation.

3.2 Evaluation of EDN control proteins

Purified endogenous EDN, from commercial suppliers Diagnostic Development and Athens, ran with an apparent molecular weight of 17 kDa in SDS-PAGE in the presence of reducing agents; the Coomassie staining was more intense with the Athens protein (Figure 1). Both samples were claimed to be >95% pure and our data were consistent with this. Further characterisation of these proteins by analytical size exclusion chromatography revealed that the Athens EDN was predominately monomeric. However, the Diagnostic Development sample contained aggregated EDN, which eluted from the Sephadex column first due to the increased molecular weight (Figure 2). We tested the reactivity of these

endogenous EDN samples in the ALPCO ELISA by preparing different concentrations using the absorbance at 280_{nm} and an extinction coefficient of 1.6. Relative to the ALPCO assay calibrators there was a slight tendency for the ALPCO assay to over determine the Diagnostic Development concentration (1.1097 x spike concentration) and under determine the Athens concentration (0.9285 x spike concentration) (Figure 3). The recombinant EDN-GST fusion and His₆-SUMO-EDN₂₈₋₁₆₁ proteins did not react in the ALPCO assay even at concentrations up to 530 ng/mL.

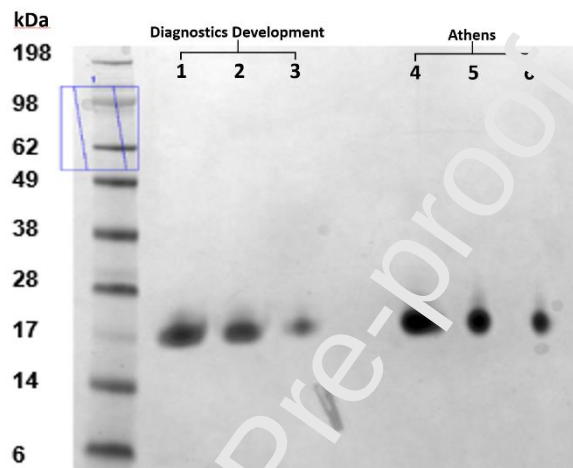


Figure 1. SDS-PAGE analysis of purified endogenous EDN. Lanes 1 and 4 contain 3.50 μ g protein; lanes 2 and 5 contain 1.75 μ g; lanes 3 and 6 contain 0.88 μ g.

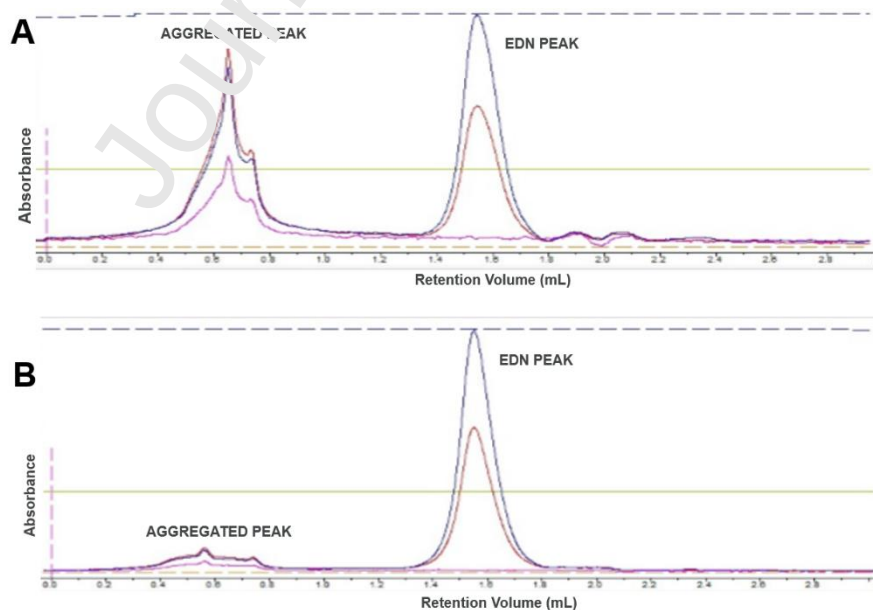


Figure 2. Analytical size exclusion chromatography of purified endogenous EDN. (A) Diagnostics Development EDN (B) Athens Research & Technology EDN. Red trace 260_{nm} and blue trace Absorbance 280_{nm}.

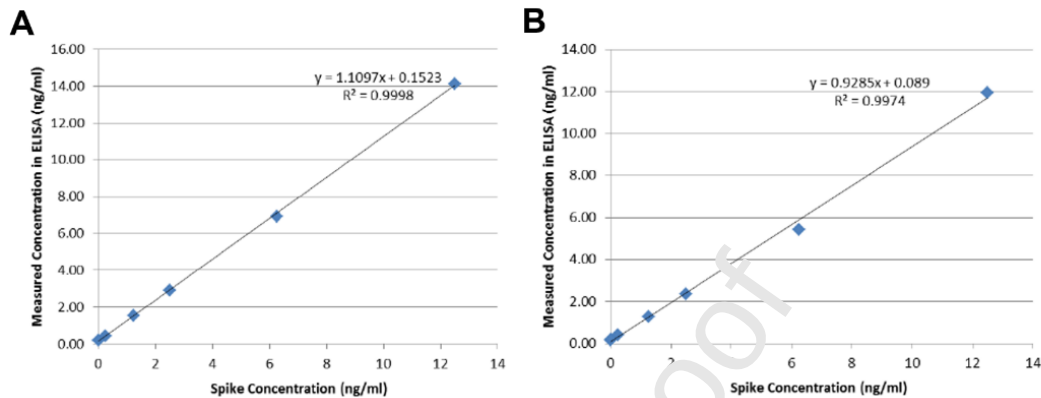


Figure 3. ALPCO ELISA quantification of purified endogenous EDN protein. Linear regression analysis of the quantification of (A) Diagnostics Development EDN (B) Athens EDN by ALPCO ELISA. Values are means of duplicates for each EDN concentration.

3.3 Commutability of EDN ELISAs

39 individual EDN serum samples were measured in each of the four commercial ELISAs. To ensure that the samples reflected clinical practice we used only serum with a range of endogenous EDN levels and avoided spiked samples. The quantification of serum EDN in the ALPCO and MBL immunoassays was highly commutable with a coefficient of determination (R^2) of 0.9195 and $p < 0.0001$ (Figure 4A). For method combinations including the LSBio or CUSABIO assays the R^2 values were found to be in the range 0.000025-0.01297 (Figures 4B-F). These results indicate that both the LSBio and CUSABIO lack serum EDN commutability with the other assays. Different patterns of assay reactivity were seen for EDN samples from healthy and asthma subjects (Figure 4G).

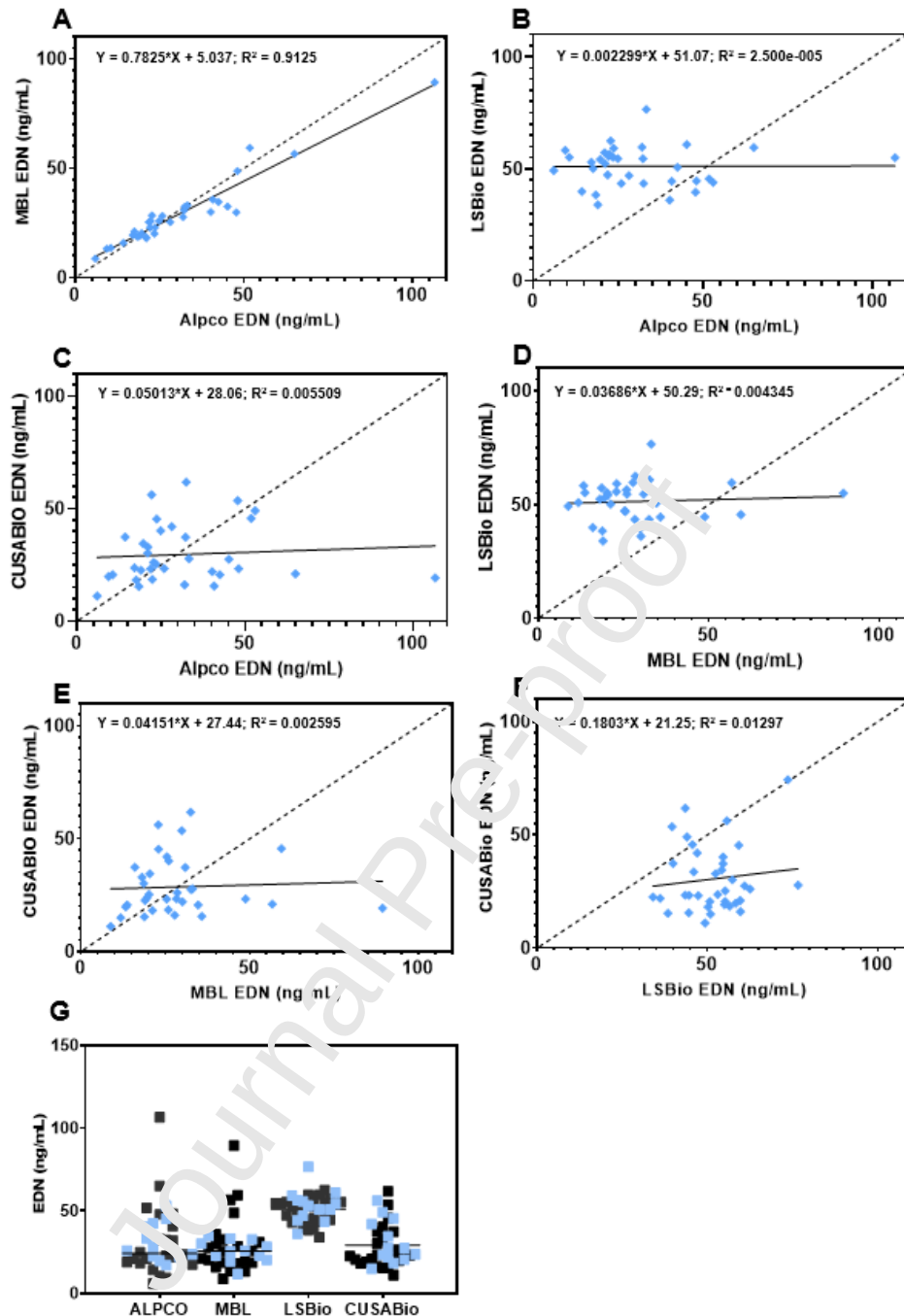


Figure 4. Commutability of serum EDN ELISAs. A-F: Linear regression analysis (solid line) and line of identity (dashed). G: Scatter plots of asthma (■) and healthy subject results (■). Values are means of duplicate samples measured in each assay.

4 Discussion

In this study we established the key analytical performance characteristics of the ALPCO EDN ELISA. Following the manufacturer's instructions, and with no additional optimisation, we were able to achieve high precision with serum samples and established

that the assay has a broad analytical measuring range that can cover both normal and disease EDN levels (Rutten et al, 2021). We successfully tested dilutional linearity up to 2560 fold sample dilution however, it may be possible to extend this further if a broader measuring range is required. Recombinant EDN fusion proteins failed to react in the ALPCO ELISA, possibly due to their lack of native glycosylation or the steric hinderance caused by the glutathione-s-transferase and His₆ purification tags. However, both the Athens and Diagnostics Development (DD) endogenous proteins, highly purified from buffy coat from anticoagulated blood, worked well in the ELISA with only marginal differences in signal, despite SEC indicating some aggregates in the DD sample. One potential disadvantage of endogenous EDN, and its possible future use as reference material, is the requirement to process large volumes of donated blood. This may possibly be circumvented by the use of blood from hyper-eosinophilic patients or via cultured eosinophil derived cell lines.

Multiple commercial ELISAs were evaluated and using a range of serum EDN levels we determined that the ALPCO and MBL ELISAs were commutable based on the linear regression analysis. The slope of the regression analysis indicated that the MBL assay under-quantified EDN relative to ALPCO. This is quite common when comparing different assays but can usually be overcome when the assays are aligned using the same assay reference materials. The MBL assay has also previously been shown to be commutable with the BioTracer™ K-EDN ELISA Kit, both of which utilise endogenous human EDN standards (Kim et al., 2017). Neither the LSBio or the CUSABio assays were commutable with any of the other assays evaluated at the serum EDN concentrations tested (6.0-106.6 ng/mL by ALPCO). The instructions for use for LSBio indicate that the standards supplied in the kit are recombinant in nature which may account for the lack of correlation. Additionally, the CUSABio analytical measuring range is reported as 0.078-5 ng/mL potentially making it incompatible with the EDN levels evaluated here. It may be more appropriate for testing EDN levels at the low end of the normal reference range, determined as 1.98 to 26.10 ng/mL using ALPCO (Rutten et al., 2021).

Since the development of the first EDN ELISA in 2000, by a Japanese research group, many commercial EDN ELISAs have become available. These ELISAs offer the opportunity to quickly, easily and precisely measure serum EDN. However, the accuracy and commutability of such assays is highly dependent on how the antibodies are generated and the materials used to generate the standard curve. Our study shows that not all EDN immunoassays are equivalent and care should be taken when comparing datasets. We would strongly advocate the establishment of EDN reference materials based on endogenous protein from blood or a suitable human cell line to standardise immunoassay measurements.

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Disclosures

This study was sponsored by AstraZeneca. Authors conflicts of interest are as follows: Bert Rutten, Simon Young, Sarah Aldridge, Rick Davies, Mark Fidock, Paul Newbold, Adam Platt and Glen Hughes were employees of the sponsor AstraZeneca at the time the study was conducted. Dave Singh has received personal fees from Aerogen, AstraZeneca, Boehringer Ingelheim, Chiesi, Cipla, CSL Behring, Epiendo, Genentech, GlaxoSmithKline, Glenmark, Gossamerbio, Kinaset, Menarini, Novartis, Pulmatrix, Sanofi, Synairgen, Teva, Theravance and Verona.

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