







Proteomics of serum extracellular vesicles identifies a novel COPD biomarker, fibulin-3 from elastic fibres

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ABSTRACT There is an unmet need for novel biomarkers in the diagnosis of multifactorial COPD. We applied next-generation proteomics to serum extracellular vesicles (EVs) to discover novel COPD biomarkers.

EVs from 10 patients with COPD and six healthy controls were analysed by tandem mass tag-based non-targeted proteomics, and those from elastase-treated mouse models of emphysema were also analysed by non-targeted proteomics. For validation, EVs from 23 patients with COPD and 20 healthy controls were validated by targeted proteomics.

Using non-targeted proteomics, we identified 406 proteins, 34 of which were significantly upregulated in patients with COPD. Of note, the EV protein signature from patients with COPD reflected inflammation and remodelling. We also identified 63 upregulated candidates from 1956 proteins by analysing EVs isolated from mouse models. Combining human and mouse biomarker candidates, we validated 45 proteins by targeted proteomics, selected reaction monitoring. Notably, levels of fibulin-3, tripeptidyl-peptidase 2, fibulin-1, and soluble scavenger receptor cysteine-rich domain-containing protein were significantly higher in patients with COPD. Moreover, six proteins; fibulin-3, tripeptidyl-peptidase 2, UTP-glucose-1-phosphate uridylyl transferase, CD81, CD177, and oncoprotein-induced transcript 3, were correlated with emphysema. Upregulation of fibulin-3 was confirmed by immunoblotting of EVs and immunohistochemistry in lungs. Strikingly, *fibulin-3* knockout mice spontaneously developed emphysema with age, as evidenced by alveolar enlargement and elastin destruction.

We discovered potential pathogenic biomarkers for COPD using next-generation proteomics of EVs. This is a novel strategy for biomarker discovery and precision medicine.



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This study identified novel biomarkers for COPD using next-generation proteomics of serum extracellular vesicles. Notably, the expression of fibulin-3 is correlated with lung function and emphysema. This could be useful for personalised medicine. <https://bit.ly/2JfRCgk>

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Introduction

COPD remains a leading cause of morbidity and mortality worldwide [1] and is a clinically heterogeneous disease composed of variable degrees of airflow obstruction, emphysema, and small airway thickening [2–4]. Moreover, COPD is often complicated by asthma and accompanied by several comorbidities, such as hypertension, hyperlipidaemia, and atherosclerosis. In addition, COPD has recently been regarded as an accelerated senescence lung disease [5]. This heterogeneous COPD is still diagnosed and treated based on lung function rather than the underlying pathobiological mechanisms; however, several potential biomarkers lack sensitivity and specificity despite large-scale studies [6–8]. Although emphysema is closely associated with the destruction of alveolar elastic fibres by elastolytic proteases, none of its biomarkers reflects its pathology.

Given the current progress in next-generation sequencing and mass spectrometry, considerable attention has been paid to both omics approaches and biomarker discovery for clarifying these heterogeneous diseases [2, 3, 9]. However, despite accumulating evidence from genomics and transcriptomics, the application of proteomics has been limited [10–12]. For example, by elaborating conventional gel-based proteomics of serum, prothrombin was identified to discriminate COPD from asthma and healthy controls [13], although it seems to be far from satisfactory. Coupled with developments in sample preparation and computational analyses, mass spectrometry (MS)-based proteomics, such as labelled quantitative proteomics and targeted proteomics, has enabled us to accurately quantitate small amounts of protein reproducibly [14–16]. Nevertheless, the application of such next-generation proteomics has rarely been utilised for respiratory diseases [3, 8].

Although a variety of biomarker resources are available, peripheral blood has been recognised as an ideal source because of the reproducibility of measurement and feasibility of access. However, approximately 22 proteins such as albumin, immunoglobulins, and complement factors comprise 99% of all serum proteins, masking small amounts of biomarker candidates when measured by conventional proteomics [17]. Extracellular vesicles (EVs) are small membrane-bound vesicles of endocytic origin that are released from most cell types. EVs have pleiotropic functions, including immune response, antigen presentation, and intercellular communication [18, 19]. As EVs are surrounded by a lipid bilayer membrane and are easily accessible from peripheral blood, they may contain a wide array of biomarkers. In fact, in multiple malignant diseases, EVs have enabled clarification of pathogenesis, diagnosis, and therapeutic strategies [20, 21]. Notably, polymorphonuclear leukocyte-derived elastase isolated from EVs in bronchoalveolar lavage fluid of patients with COPD induced emphysema in mice, suggesting that EVs can transfer pathogenic messages in COPD [22].

Although confirmation of phenotypic differences should be made at the proteomic level, most research on EVs in the respiratory field has focussed on mRNA and miRNA, as it is challenging to analyse small quantities of EV proteins [23]. Here, we applied the latest proteomic strategies to assess serum EVs to find novel biomarkers for personalised medicine in COPD.

Methods

Methodological details are available in the supplementary material.

Sample collection and approval

All serum samples were collected in Osaka University Hospital and stored at -80°C until analysis. Approval was obtained from the Osaka University Graduate School of Medicine Institutional Review Board; all patients gave written informed consent to participate in the study. Mouse experiments were approved by the Osaka University's Animal Care and Use Committee, and they were performed following the Declaration of Helsinki on animal care.

Study design

The discovery phase comprised six healthy controls and 10 COPD patients (supplemental table E1); non-targeted proteomics with tandem mass tag (TMT)-labels of serum EVs were conducted [14]. To identify

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additional biomarker candidates from simple mouse models, serum samples were collected from 10 mice after 21 days of treatment with intranasal elastase or PBS as previously reported [24]. Serum samples from each group were pooled, and non-targeted proteomics with isobaric tags for relative and absolute quantitation (iTRAQ) was conducted as previously reported [15]. The validation phase comprised 20 healthy controls and 23 patients with COPD. Candidate proteins from both human and mouse studies were chosen for validation and quantified by selected reaction monitoring (SRM) (figure 1a).

Patient characteristics

Subjects in the COPD group were current or ex-smokers with compatible symptoms, a diagnosis of COPD by the physician, and incompletely reversible airflow obstruction (forced expiratory volume/forced vital capacity <0.7 post-bronchodilator) according to the Global Initiative for Chronic Obstructive Lung Disease, and patients with asthma were excluded.

Sample preparation for proteomics of extracellular vesicles

EVs were isolated from serum by ultracentrifugation with differential cushions as described previously [14], and the protocols were in accordance with the most recent guidelines (MISEV 2018) [19]. This study was registered in EV-track (EV200079). Isolated EVs were confirmed using transmission electron microscopy (TEM). Size distributions and numbers were confirmed by nanoparticle tracking analysis NanoSight (Malvern Instruments, Malvern, UK) for humans and by tunable resistive pulse sensing qNano (Meiwafosis Co. Ltd., Tokyo, Japan) for mice [22, 25]. The processing of the EV proteins for proteomic analyses was performed as indicated in the Supplemental material.

Non-targeted proteomics

In the discovery phase, quantitative proteomics using isobaric chemical labelling was performed using liquid chromatography–tandem mass spectrometry analyses on hybrid quadrupole-Orbitrap mass spectrometers (Thermo Fisher Scientific, Bremen, Germany). TMT labels were used in the human discovery phase, and iTRAQ labels were used for mice [14, 15]. Additionally, subsequent database searches were conducted.

Targeted proteomics: selected reaction monitoring

In the validation phase, protein abundance was measured with SRM performed on a TSQ-Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific) as previously reported [14–16]. Each protein was confirmed by two unique peptides. The SRM data were analysed by Skyline software (MacCoss Lab Software, Seattle, WA, USA) [26]. The peak area ratios of endogenous light (L) peptides and their heavy (H) isotope-labelled internal standards were used for accurate quantification. The peptide light/heavy ratios were \log_2 transformed and centred at the median.

Statistical analyses

The following statistical analyses were performed with JMP Pro v. 14.3.0 (SAS Institute, Cary, NC, USA): t-test, Welch's t-test, Spearman's rank correlation coefficients, logistic regression analysis, and analysis of covariance. A p-value <0.05 was considered statistically significant.

Results

Discovery of novel biomarkers for COPD by proteomics

Based on TEM, most of the vesicles isolated from healthy controls and patients with COPD similarly expressed a general EV marker protein, CD9 on their surfaces (figure 1b). Additionally, isolated EVs were positive for CD63, CD9, and flotillin-1, and negative for calnexin and apolipoprotein A1 (figure 1c). By nanoparticle tracking analysis, the diameter of serum EVs from both groups were smaller than 200 nm, and indistinguishable in size and number (figure 1d–f).

Using non-targeted proteomics, we identified as many as 406 proteins in EVs from <20 μ L serum from healthy controls and patients with COPD (figure 2a). Notably, 32 proteins were significantly upregulated, whereas 43 proteins were downregulated in patients with COPD compared with those in healthy controls (table 1 and E2). Identified proteins were present in the extracellular space (38.9%), cytoplasm (29.6%), and plasma membrane (23.9%) (supplemental figure E1a).

To verify the strength of EV analysis over that of serum, we further analysed serum by non-targeted proteomics after immunodepletion of highly abundant proteins (supplemental material). As expected, the number of proteins identified from EVs was higher than that from serum. Among the 406 proteins identified in EVs, 155 were also found in serum, whereas 251 were unique to EVs (figure E1b). While fractions from the plasma membrane and the cytoplasm were more abundant in EVs, proteins, regarded as noise from the point of proteomics [17], were by far richer in serum than in EVs (fig. E1a and c).

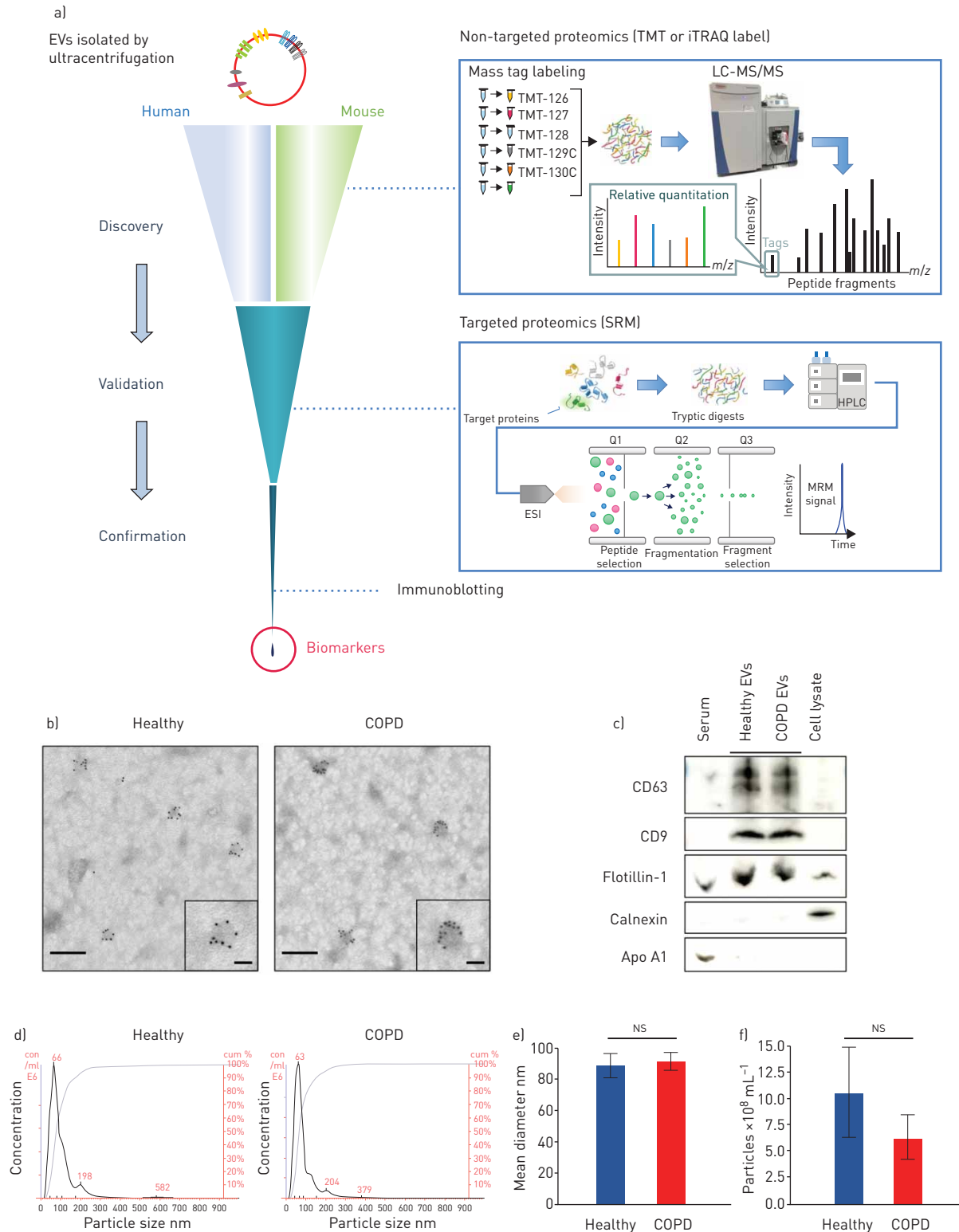


FIGURE 1 Discovery of novel biomarkers for COPD. a) In the discovery phase, serum extracellular vesicles (EVs) isolated by ultracentrifugation from patients with COPD and healthy controls were analysed. Simultaneously, serum EVs from elastase-treated mouse models were similarly analysed. In the validation phase, biomarker candidates from both humans and mice were further quantified by targeted proteomics, selected reaction monitoring (SRM). Finally, the biomarkers were confirmed by immunoblotting. b) Transmission electron microscopic images of serum EVs from a healthy control and a patient with COPD; immunogold labelling with CD9. Scale bar=200 nm, scale bar in the inset=50 nm. c) Serum EVs were compared with serum and Jurkat cell lysate by immunoblotting of CD63, CD9, flotillin-1, calnexin, and apolipoprotein (Apo) A1. d) Representative figures of the particle size distribution curve of serum EVs from a healthy control and a patient with COPD using NanoSight. e, and f) No differences were seen in the mean diameters or numbers of serum EVs analysed using NanoSight from healthy controls and patients with COPD. Error bars indicate mean \pm SD. NS: not significant.

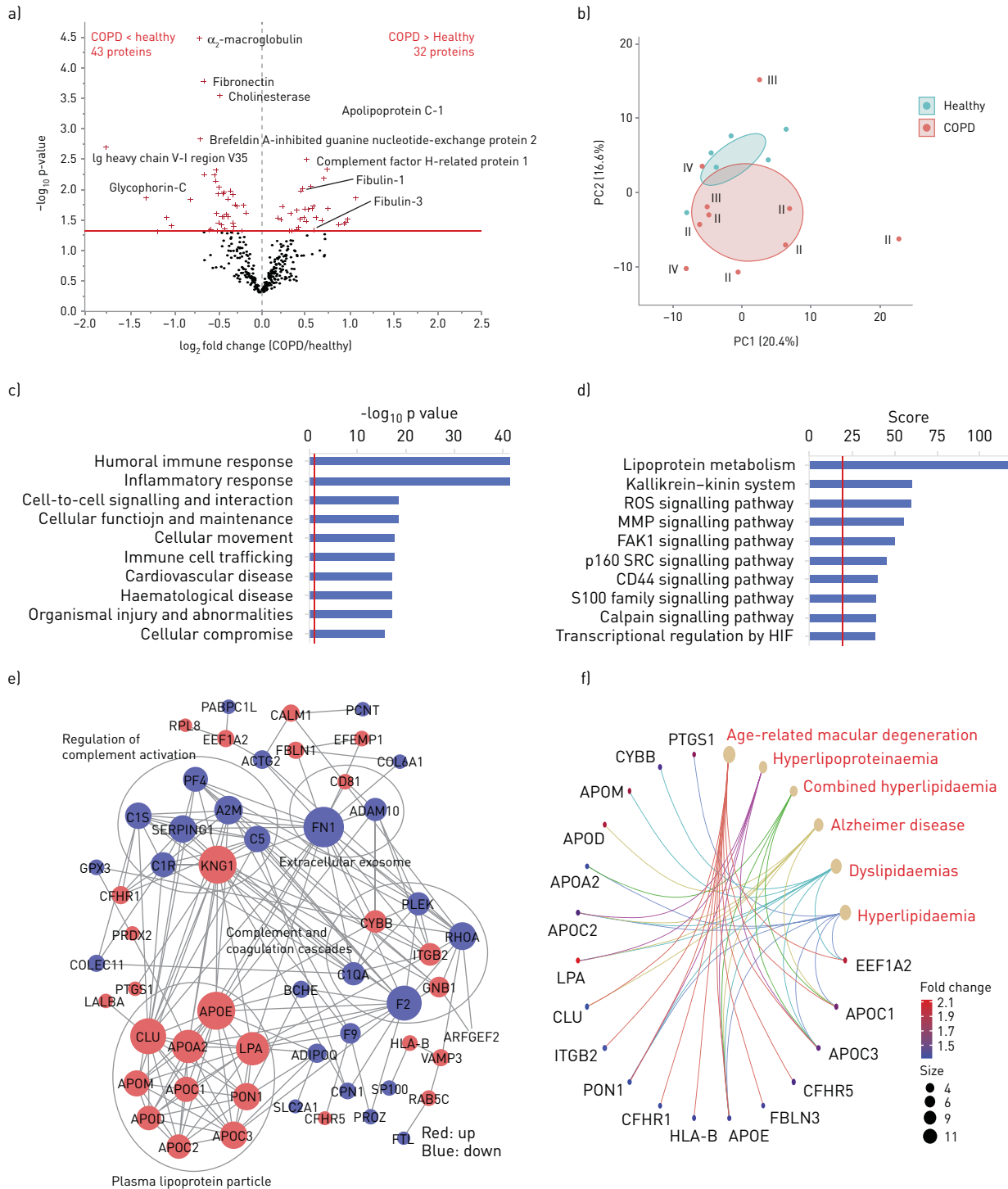


FIGURE 2 Proteomic profile of serum extracellular vesicles (EVs) reflects COPD characteristics. **a)** A volcano plot of all identified 406 serum EV proteins by non-targeted proteomic analyses in patients with COPD and healthy controls. Thirty-two proteins were significantly upregulated, and 43 proteins were significantly downregulated in EVs of patients with COPD compared with those of healthy controls. Red line, p -value=0.05. **b)** Healthy controls and patients with COPD were moderately separated in principal-component analysis using all identified proteins in EVs by non-targeted proteomics. II, III, and IV GOLD COPD stages. **c)** Top 10 diseases and bio functions determined by ingenuity pathway analysis to be over ($\times 1.2$ or more) and under ($\times 0.8$ or less) represented by non-targeted proteomic analyses in serum EVs of patients with COPD compared with those of healthy controls. Red line, p -value=0.05. **d)** Top 10 pathways determined by KeyMolnet to be over ($\times 1.2$ or more) and under ($\times 0.8$ or less) represented by non-targeted proteomic analyses in serum EVs of patients with COPD compared with those of healthy controls. Red line, score by hypergeometric distribution, cut-off value=20. **e)** A protein-protein interaction network constructed with Cytoscape based on the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database reveals complex interactions between these proteins. The significantly upregulated proteins are coloured in red and downregulated proteins are in blue. The node size reflects the number of connected edges. **f)** Top six diseases determined by disease enrichment analysis to be over- ($\times 1.2$ or more) and under- ($\times 0.8$ or less) represented by non-targeted proteomic analysis in serum EVs of patients with COPD compared with those of healthy controls.

In principal-component analysis, healthy controls and patients with COPD were moderately separated (figure 2b). Notably, ingenuity pathways analysis (IPA) included humoral immune response, inflammatory response, cell-to-cell signalling, and cellular movement, which reflected various aspects of inflammation in COPD (figure 2c). In addition, reactive oxygen species, matrix metalloproteinase, and S100 family signalling pathways, regarded as critical components in COPD pathology, were upregulated using KeyMolnet (figure 2d). Furthermore, protein–protein interaction analyses revealed interconnected interactions of significantly up- and downregulated proteins (figure 2e). By disease enrichment analysis, correlations between these molecules and COPD comorbidities such as dyslipidaemia were confirmed (figure 2f). Using Ward’s hierarchical cluster analysis, patients with COPD were divided into two groups by significantly up- and downregulated proteins, indicating that these biomarker candidates might be helpful in endotyping COPD (figure E2).

Non-targeted proteomics of serum EVs in mouse models

To obtain biomarkers that reflect alveolar destruction of the most crucial COPD phenotype emphysema, we utilised an elastase-treated mouse model of emphysema rather than a *cigarette smoke model* (figure 3a).

TABLE 1 Significantly upregulated proteins in serum extracellular vesicles of patients with COPD compared with those of healthy controls by t-test

Protein ID	Gene name	Protein name	Healthy	COPD	C/H	p-value
P02654	<i>APOC1</i>	Apolipoprotein C-I	0.74	1.29	1.75	<0.001
Q03591	<i>CFHR1</i>	Complement factor H-related protein 1	0.74	1.06	1.43	0.003
P02655	<i>APOC2</i>	Apolipoprotein C-II	0.75	1.26	1.68	0.005
Q9BXR6	<i>CFHR5</i>	Complement factor H-related protein 5	0.77	1.26	1.64	0.006
Q95365	<i>HLA-B</i>	HLA class I histocompatibility antigen, B-38 α -chain	0.81	1.20	1.48	0.009
P23142	<i>FBLN1</i>	Fibulin-1	0.79	1.10	1.39	0.010
P05107	<i>ITGB2</i>	Integrin β -2	0.77	1.06	1.37	0.010
P08519	<i>LPA</i>	Apolipoprotein(a)	0.75	1.57	2.11	0.014
Q6ZN30	<i>BNC2</i>	Zinc finger protein basonuclin-2	0.84	1.27	1.51	0.018
P60033	<i>CD81</i>	CD81 antigen	0.95	1.12	1.18	0.019
P02656	<i>APOC3</i>	Apolipoprotein C-III	0.73	1.24	1.69	0.020
P02649	<i>APOE</i>	Apolipoprotein E	0.81	1.21	1.49	0.020
P14209	<i>CD99</i>	CD99 antigen	0.84	1.21	1.44	0.021
P01042	<i>KNG1</i>	Kininogen-1	0.83	1.17	1.41	0.021
A1L4H1	<i>SSC5D</i>	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D	0.91	1.20	1.32	0.021
P32119	<i>PRDX2</i>	Peroxiredoxin-2	0.89	1.12	1.26	0.025
P27169	<i>PON1</i>	Serum paraoxonase/arylesterase 1	0.81	1.13	1.40	0.028
Q8WWZ8	<i>OIT3</i>	Oncoprotein-induced transcript 3 protein	0.72	1.11	1.54	0.029
P62158	<i>CALM1</i>	Calmodulin	0.89	1.02	1.14	0.029
Q05639	<i>EEF1A2</i>	Elongation factor 1- α 2	0.65	1.28	1.98	0.031
P00709	<i>LALBA</i>	α -lactalbumin	0.87	1.18	1.36	0.031
P62873	<i>GNB1</i>	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β -1	0.87	1.01	1.17	0.031
P04839	<i>CYBB</i>	Cytochrome b-245 heavy chain	0.65	1.04	1.60	0.032
P02652	<i>APOA2</i>	Apolipoprotein A-II	0.85	1.21	1.42	0.033
P05090	<i>APOD</i>	Apolipoprotein D	0.70	1.36	1.94	0.034
Q95445	<i>APOM</i>	Apolipoprotein M	0.50	0.96	1.92	0.036
P23219	<i>PTGS1</i>	Prostaglandin G/H synthase 1	0.67	1.24	1.84	0.037
P10909	<i>CLU</i>	Clusterin	0.85	1.14	1.34	0.042
P62917	<i>RPL8</i>	60S ribosomal protein L8	0.84	1.12	1.32	0.045
Q12805	<i>EFEMP1</i>	EGF-containing fibulin-like extracellular matrix protein 1 (fibulin-3)	0.92	1.39	1.52	0.047
P51148	<i>RAB5C</i>	Ras-related protein Rab-5C	0.87	1.10	1.27	0.048
Q15836	<i>VAMP3</i>	Vesicle-associated membrane protein 3	0.96	1.20	1.25	0.048

Healthy and COPD: mean quantitative values of proteins in extracellular vesicles of healthy controls and patients with COPD, respectively. C/H: mean quantitative values of proteins in extracellular vesicles of patients with COPD over those of healthy controls.

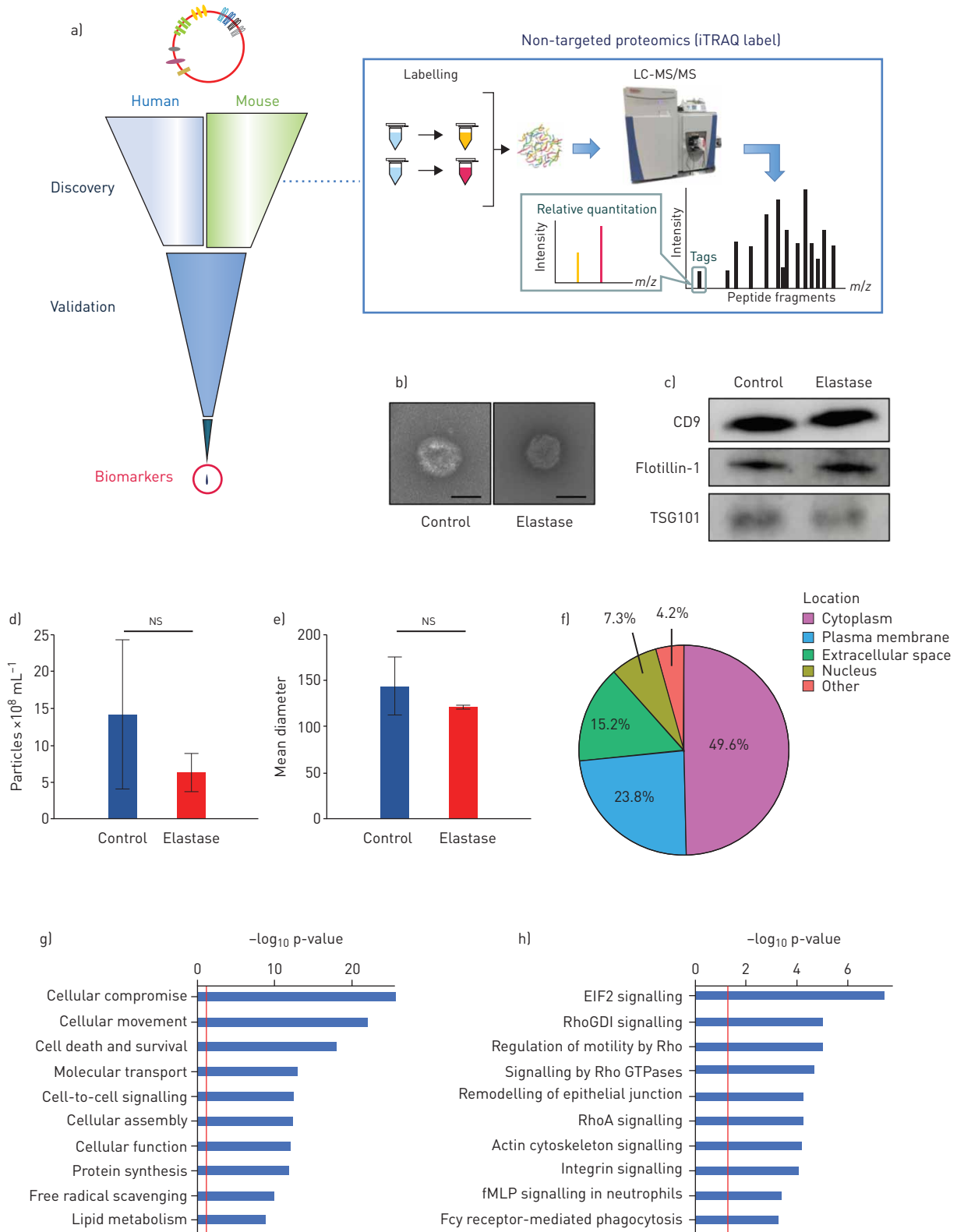


FIGURE 3 The general strategy to discover novel biomarkers from serum extracellular vesicles (EVs) in mice. **a)** A flowchart of non-targeted proteomics with iTRAQ of mouse serum EVs. **b)** Transmission electron microscope images of serum EVs from a PBS- and an elastase-treated mouse. Scale bar=100 nm. **c)** Immunoblotting results of serum EVs from a PBS- and an elastase-treated mouse; no differences were observed in the EV marker proteins, CD9, flotillin-1, or TSG101. **d)** and **e)** No differences were seen in the number or mean diameter of serum EVs analysed using qNano from PBS- and elastase-treated mice. Error bars indicate mean \pm SD. NS: not significant. **f)** Localisation of all identified proteins by ingenuity pathway analysis. **g)** and **h)** Top 10 molecular and cellular functions and canonical pathways determined by ingenuity pathway analysis to be over-represented ($\times 1.5$ or more) by the non-targeted proteomic analysis. Red line, p -value=0.05.

Isolated EVs from mice were confirmed using TEM, immunoblotting, and qNano. Consistent with human results, EVs from control and elastase-treated mice were comparable in number and size (figure 3b–e). From serum EVs of mice, we obtained 1956 proteins as determined by iTRAQ labelled non-targeted proteomics. Among them, 63 proteins were upregulated by two-fold or more, and 16 proteins were downregulated by half or less (tables E3 and E4). Identified proteins were localised in the cytoplasm (49.6%), plasma membrane (23.8%), and extracellular space (15.2%) (figure 3f). Similar to the findings in the IPA of patients with COPD, molecular and cellular functions included cellular movement and cell-to-cell signalling, which reflected various aspects of inflammation in COPD (figure 3g). Furthermore, inflammatory responses such as EIF2 signalling and remodelling of epithelial junctions were listed as the most likely canonical pathways (figure 3h). Of note, commonly upregulated biomarker candidates in mice and humans consisted of 15 proteins, including fibulin-1, fibulin-3, integrin β 2, and peroxiredoxin-2 (figure E3).

Validation of COPD biomarkers by targeted proteomics; selected reaction monitoring

Sixteen proteins were identified as biomarker candidates in COPD patients, and 29 proteins were added from the mouse models; therefore, 45 proteins were selected for validation (figure 4a, table E5). We utilised SRM, both quantitatively and reproducibility have been established in our laboratory, to validate these biomarker candidates [14–16]. In the validation phase, there were more men than women in the COPD patient group, and patients with COPD were older and had a more extended history of smoking than controls (table E6).

Notably, expressions of fibulin-3, tripeptidyl-peptidase (TPP) 2, fibulin-1, and soluble scavenger receptor cysteine-rich domain-containing protein (SRCRL) were significantly upregulated in serum EVs of patients with COPD (figure 4b), and upregulation was confirmed by two kinds of unique peptides in both fibulin-3 and TPP2. Although both fibulin-1 and -3 were detected in serum by ELISA, no differences in their concentrations were observed (figure E4). By receiver operating characteristic curve analysis, the areas under the curve (AUC) of fibulin-3, TPP2, fibulin-1, and SRCRL were 0.73, 0.67, 0.71, and 0.72, respectively (figure 4c). In combining these four proteins as multiple markers using logistic regression analysis, the AUC was further increased to 0.92 (figure 4d). In comparing only ex- and current smokers, 22 patients with COPD and nine healthy controls, to evaluate the influence of smoking status, all four proteins were still significantly upregulated in patients with COPD (data not shown).

Novel biomarkers were correlated with respiratory function and emphysema

To further characterise the clinical potential of these biomarkers, we analysed the correlation between lung function and low-attenuation areas in computed tomography (CT) images. Notably, seven proteins; chloride intracellular channel protein 1, TPP2, fibulin-3, EMILIN-1, S100A9, oncoprotein-induced transcript 3 (OIT3), and thrombospondin-4 were significantly correlated with percentage predicted forced expiratory volume in 1 s (FEV_1) (figure 5a). Moreover, six proteins (TPP2, UTP-glucose-1-phosphate uridylyl transferase, CD81, fibulin-3, CD177, and OIT3) were correlated with low-attenuation areas in CT (figure 5b). Of note, TPP2, chloride intracellular channel protein 1, EMILIN-1, S100A9, thrombospondin-4, CD177, and UTP-glucose-1-phosphate uridylyl transferase, which were identified only in the mouse discovery phase, were also validated in humans.

Confirmation of novel biomarkers by immunoblotting

To further confirm these results, we performed immunoblotting and immunohistochemistry. Fibulin-3 was significantly upregulated in patients with COPD, as indicated by densitometric relative quantification of the immunoblotting results (figure 6a and b). Existence of fibulin-3 in EVs isolated by commercially available size exclusion chromatography EVSecond (GL science, Tokyo, Japan) [21] were also confirmed by immunoblotting (figure E5). Moreover, fibulin-3 expression in EVs was further confirmed using TEM (figure 6c). Importantly, fibulin-3 was highly expressed in both alveolar epithelial cells and alveolar interstitial spaces in the lungs of patients with COPD (figure 6d). In addition, TPP2, fibulin-1, CD81, EMILIN-1, and OIT3 were also expressed in both alveolar epithelial cells and connective tissues in the lungs to varying degrees (figure E6). Regarding the possible involvement of fibulin-3 in the pathogenesis of COPD, CD44 and collagen were upstream and transforming growth factor- β and α 1-antitrypsin were downstream of fibulin-3 based on KeyMolnet (figure 6e).

Fibulin-3 knockout mice spontaneously develop emphysema with age.

Given that fibulin-3 plays a crucial role in elastic fibre assembly and is correlated with emphysema, we generated *fibulin-3* knockout mice. Defects of fibulin-3 in knockout mice were confirmed by immunoblotting (figure E7a). Consistent with prior reports, inguinal herniation, rectal prolapse and a large bulge at the anorectal area [27, 28] were observed in knockout mice with similar body weight (figure E7b). Knockout mouse skins were looser compared with those of wild-type mice, and knockout mouse

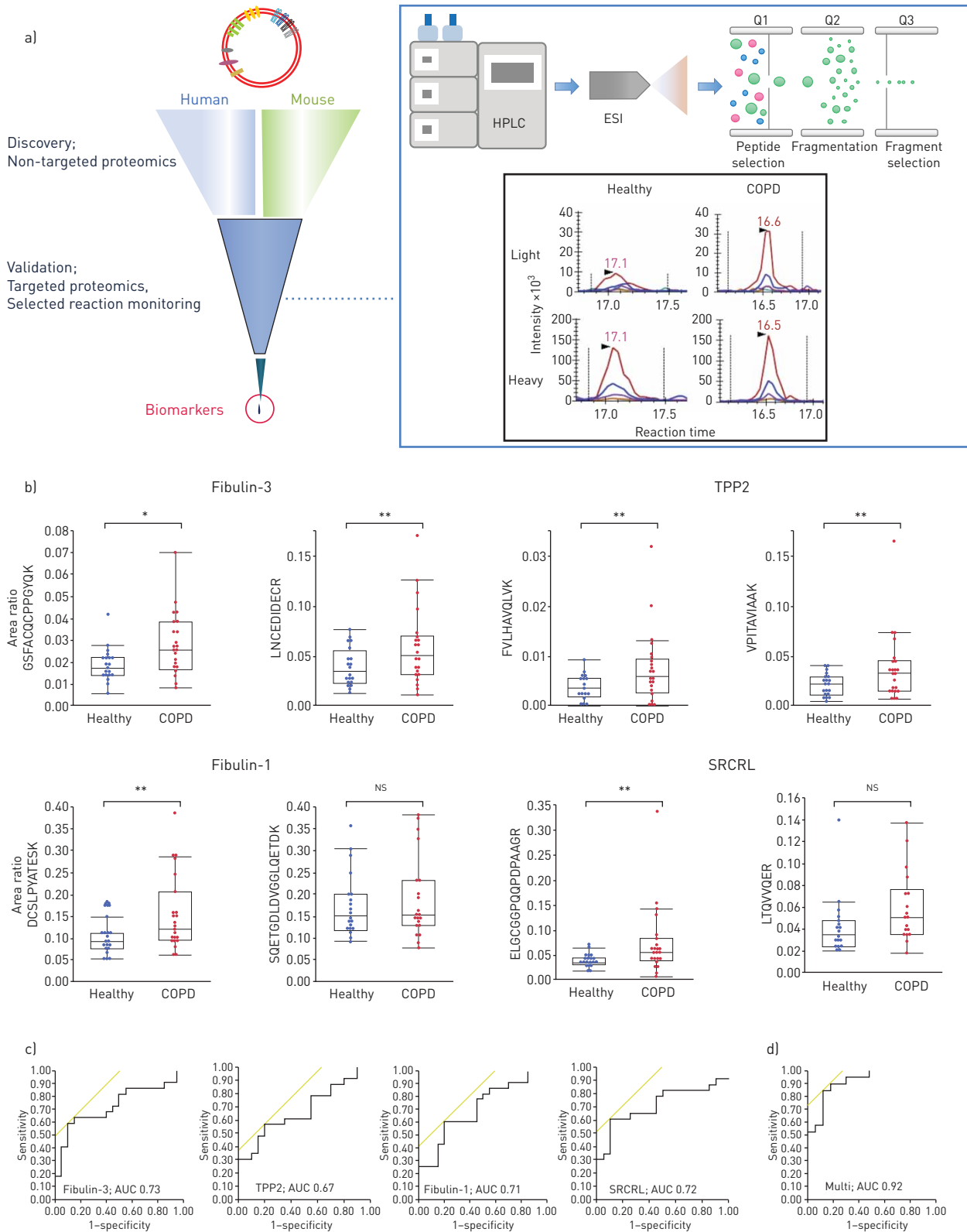


FIGURE 4 Four novel biomarkers were verified by selected reaction monitoring [SRM]. a) A flowchart of this study and representative figures of SRM. The quantitation of each endogenous protein in serum extracellular vesicles (EVs) was performed by comparing the brown area of an endogenous peptide (light) to the corresponding synthetic peptide (heavy). b) Significantly upregulated proteins in serum EVs of patients with COPD compared with those of healthy controls as determined by targeted proteomics. All proteins were quantified by two unique peptides. TPP2: tripeptidyl-peptidase 2; SRCRL: soluble scavenger receptor cysteine-rich domain-containing protein; *: $p < 0.01$; **: $p < 0.05$; ns: not significant. c) The receiver-operating characteristic (ROC) curves of four proteins listed in fig. 4b to diagnose COPD. AUC: area under the curve. d) The ROC curve of a multi-marker with four proteins listed in (c), calculated by logistic regression analysis, to diagnose COPD.

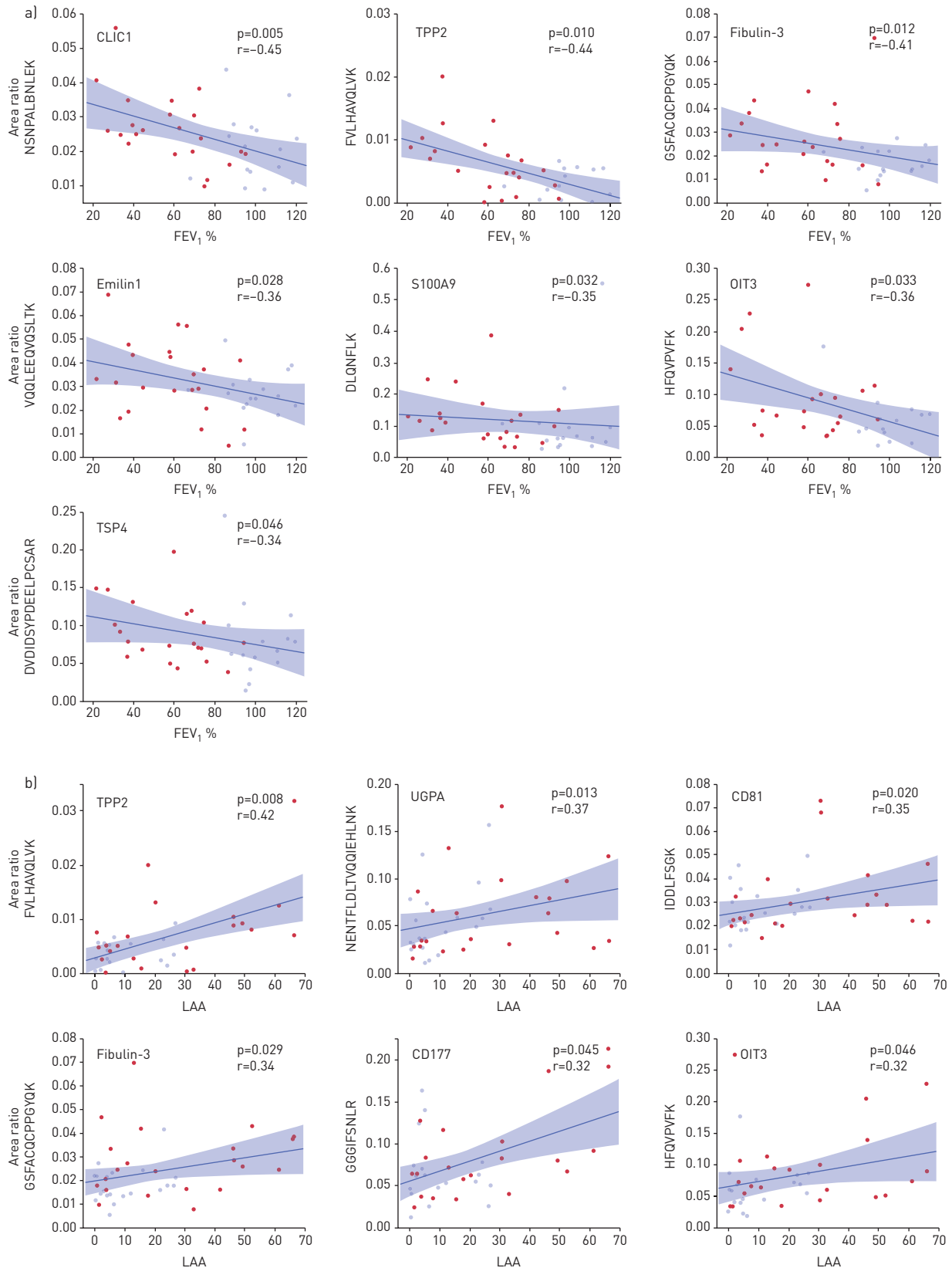


FIGURE 5 Novel extracellular vesicle (EV) biomarkers are correlated with lung function and emphysema. a) Seven proteins were significantly correlated with the percentage predicted forced expiratory volume in 1 s (FEV₁ %). Red dots represent patients with COPD; blue dots represent healthy controls. r indicates Spearman's rank correlation coefficients; CLIC: chloride intracellular channel protein; TPP: tripeptidyl-peptidase; OIT: oncoprotein-induced transcript; TSP: thrombospondin. b) Six proteins were significantly correlated with percentage lung volume with <-910 HU at full inspiration in computed tomography images. LAA: low-attenuation area; UGPA: UTP-glucose-1-phosphate uridylyl transferase.

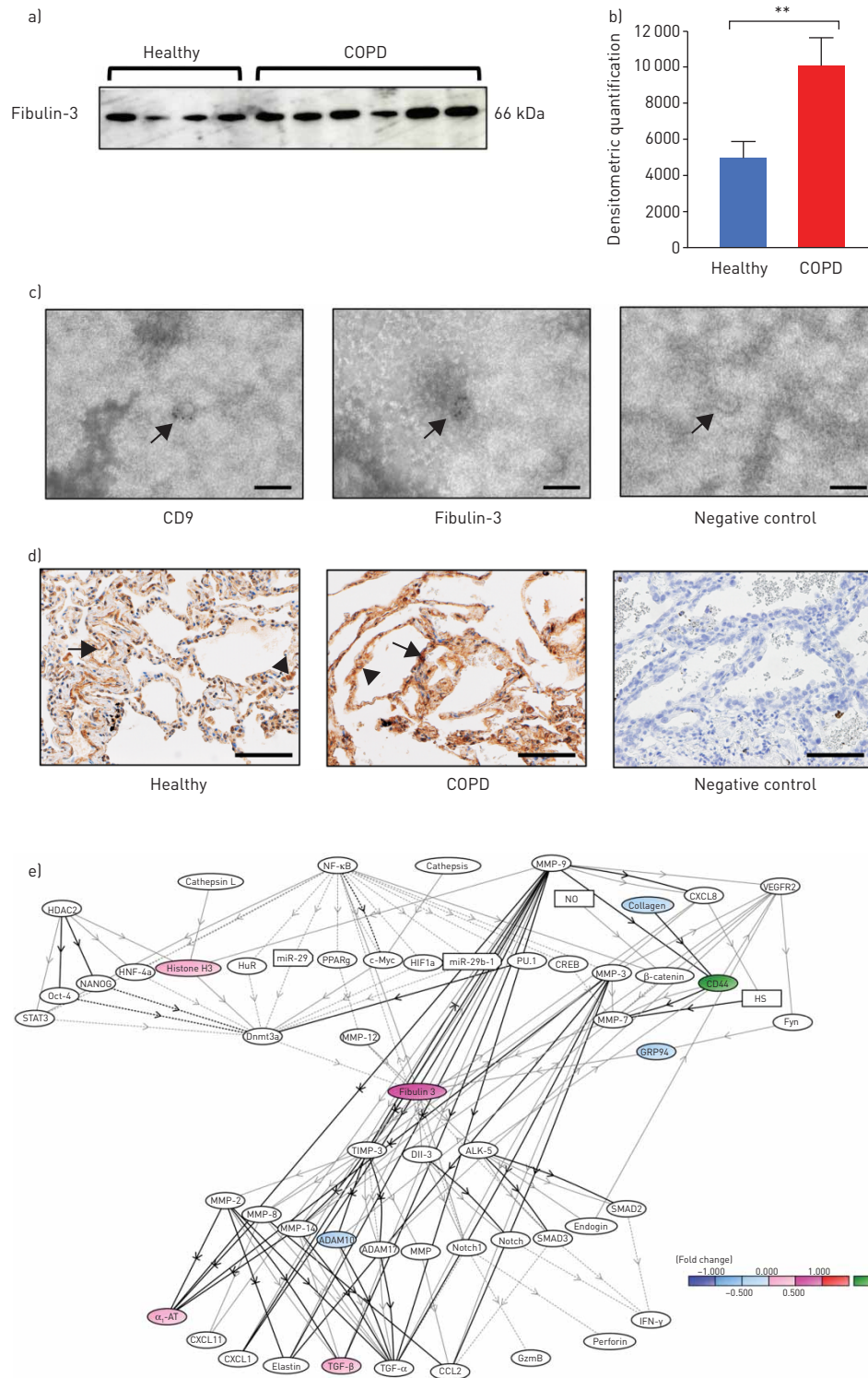


FIGURE 6 Confirmation of fibulin-3 upregulation in serum extracellular vesicles (EVs) and lungs of patients with COPD. **a)** A representative image of immunoblotting comparing fibulin-3 in serum EVs of healthy controls and patients with COPD. **b)** Densitometric analyses of the immunoblotting results in (a). Error bars indicate mean ± SEM. **: p < 0.05. **c)** The transmission electron microscopy (TEM) panels show fibulin-3 expression of EVs with anti-fibulin-3 primary antibody, and anti-IgG gold-labelled secondary antibody. Rabbit IgG was used in place of a primary rabbit polyclonal antibody of fibulin-3 as a negative control. Scale bar = 100 nm. **d)** Immunohistochemical staining of a COPD lung revealed that fibulin-3 was highly expressed in both alveolar epithelium cells (black arrow) and connective tissues (black arrowhead). Fibulin-3 was also expressed in alveolar macrophages (white arrowhead) and vessel walls (white arrow) in a healthy lung. Rabbit IgG was used in place of a primary rabbit polyclonal antibody of fibulin-3 as a negative control. Scale bar = 100 μm. **e)** Using the “start points and end-points” network search algorithm, KeyMolnet illustrated a highly complex network of targets with the most statistically significant relationships. The cluster of upregulated proteins, including fibulin-3, is highlighted by a red circle. The molecular relationships are indicated by a solid line with arrow (direct binding or activation) and solid line without arrow (complex formation). Colour set point is described in the right panel.

elastic fibres were reduced in the dermic layer (figure 7a and b). Strikingly, knockout mice showed emphysema at 22 weeks of age, but not at 12 weeks (figure 7c). However, no significant difference was observed in the cell number obtained by lung lavage (figure E7c). Although elastin was disrupted in knockout mice compared with wild-type mice, the appearance of collagen was similar in both groups (figure 7d). Of importance, elastin with irregular alignment and collagen with less density were found, presumably representing abnormal development or remodelling (figure 7e). Collectively, fibulin-3, which is linked with not only lung function but also pathogenesis, could be a novel COPD biomarker.

Discussion

There is an urgent need to develop biomarkers that enable precision medicine and improve health outcomes of patients with COPD. However, no single protein biomarker has been adopted for routine clinical use as biomarker discovery remains challenging. Although peripheral blood may be regarded as an ideal resource for biomarkers, MS-based serum proteomics is exceptionally challenging due to its broad dynamic range with miscellaneous proteins [17]. To overcome these obstacles, we focussed on serum EVs and identified new biomarkers for COPD by making full use of next-generation proteomics.

The importance of EVs, especially exosomes, has been increasingly demonstrated in cancer and immune diseases [20–22, 29]. Despite accumulating evidence from cancer biomarkers, biomarker discovery for inflammatory diseases from a proteomics approach is extremely limited. Considering that isolating EVs can reduce the complexity of serum and EVs reflect both inflammation and the pathophysiology of COPD, serum EVs could become an ideal resource for novel biomarkers in terms of liquid biopsy for personalised medicine. Although EVs have advantages such as stability, accessibility, and the propensity to harbour novel messengers, they also have disadvantages such as difficulty in both isolation and quantitation, and contamination with serum constituents [19]. In addition, analysing a small number of EV proteins is quite challenging. To overcome these hurdles, non-targeted proteomics coupled with high-performance liquid chromatography is needed [14, 15].

Here, several biomarker candidates from mouse models were verified in humans. This strategy can provide mechanistic insights into common and divergent pathways between species. Therefore, EV proteins reflected the pathophysiology of COPD in both mice and humans. Despite the identification of only a few common biomarkers, such molecules could be important for the assessment of pathogenesis and designing new therapies (figure 8). While several mouse models have been used to investigate novel biomarkers by proteomics [30], translation of identified biomarkers into clinical studies is still lacking. Verifying many biomarker candidates from mice and humans by conventional immunoblotting methods might be time-consuming and less specific. Although targeted proteomics enabled us to verify biomarker candidates efficiently and specifically without using any antibody, the application of next-generation proteomics has been limited in the respiratory field. Here, we identified novel biomarkers by combining non-targeted proteomics and targeted proteomics.

Among our novel biomarkers, extracellular matrix proteins involved in elastic fibre assembly, fibulin-1 and -3, showed higher expression in serum EVs of patients with COPD. Considering emphysema is the dominant phenotype of COPD, it is important to monitor extracellular matrix protein fragments from lung parenchyma. Desmosine, a fragment of elastic fibres in the lung, attracted attention as a biomarker for COPD, but quantification in blood requires MS and is difficult for practical use [31]. Despite the pivotal function of fibulin-5 in this family [32], no difference in the expression of fibulin-5 could be observed in either serum or EVs in this study (data not shown). Furthermore, serum fibulin-1 has been reported to be associated with COPD and fibrosis in mice and humans, and genetic inhibition of *fibulin-1* protects against cigarette smoke-induced airway fibrosis and emphysema [33]. Thus, the serum EV fibulin-1 and -3 found in this study may be new extracellular matrix proteins that indicate alveolar destruction.

In recent studies, fibulin-3, 4, and 5, a collection of matricellular proteins known as “short fibulins”, have been shown to be important components of elastic fibre assembly [34]. Fibulin-5, which has a very similar structure as fibulin-3, is reportedly upregulated in COPD lungs at the messaging and protein levels [35]. It seems paradoxical that elastin in the COPD lung must be destroyed, although this is the result of an attempt to repair the damaged lung, which is not effective [36]. The ineffective repair response in COPD might be due to the upregulation of cleaved fibulin-5 by increased serine protease in COPD lungs. The cleaved form of fibulin-5 does not promote elastic fibre assembly *in vitro*, and thus seems to be nonfunctional [37]. Intriguingly, fibulin-5 knockout mice were reported to exhibit severe emphysema [32]. There have been some similar cases, for example, surfactant protein-D or adiponectin levels increase in COPD lungs and protect them at the same time, but these knockout mice also develop emphysema [38, 39].

In contrast to other fibulins, the role of fibulin-3 (also known as EGF-containing fibulin-like extracellular matrix protein 1) in elastic fibre assembly is not understood well. According to a meta-analysis of

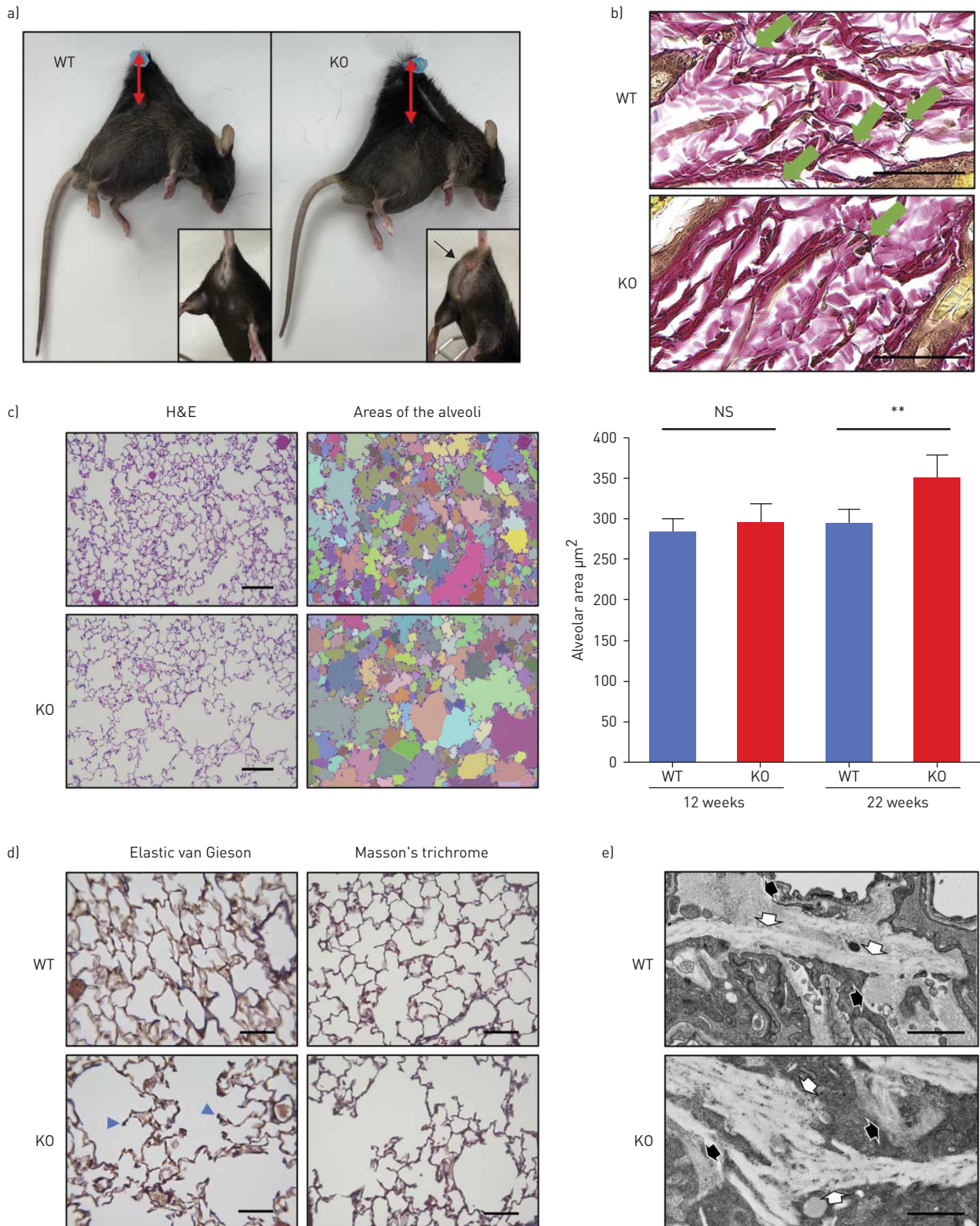


FIGURE 7 *Fibulin-3* knockout (KO) mice spontaneously developed emphysema with age. a) Looser skin compared with the wild-type (WT) mouse (red up down arrow) and a rectal prolapse and a large bulge at the anorectal area (black arrow) were observed in the 5-month-old female KO mouse. b) Elastic van Gieson stain of the dermic layer of a 5-month-old male mouse. Elastic fibres (green arrows) were reduced in KO mice compared with those of WT. Scale bar=50 μm . c) KO mice showed emphysema at 22 weeks old, but not at 12 weeks. Scale bar=100 μm . Error bars indicate mean \pm SEM. NS: not significant; **, $p < 0.05$. d) Elastin stained by Elastic van Gieson disrupted in KO mice compared with WT mice. Arrowheads indicate sparse elastin fibres in KO lungs. Collagen stained by Masson's trichrome was indistinguishable. Scale bar=50 μm . e) Transmission electron microscopy (TEM) images of WT and KO mouse lungs. Elastic fibres (white arrows) in KO mouse lungs were irregularly aligned and collagen fibres (black arrows) were less dense compared with those of WT. Scale bar=1 μm .

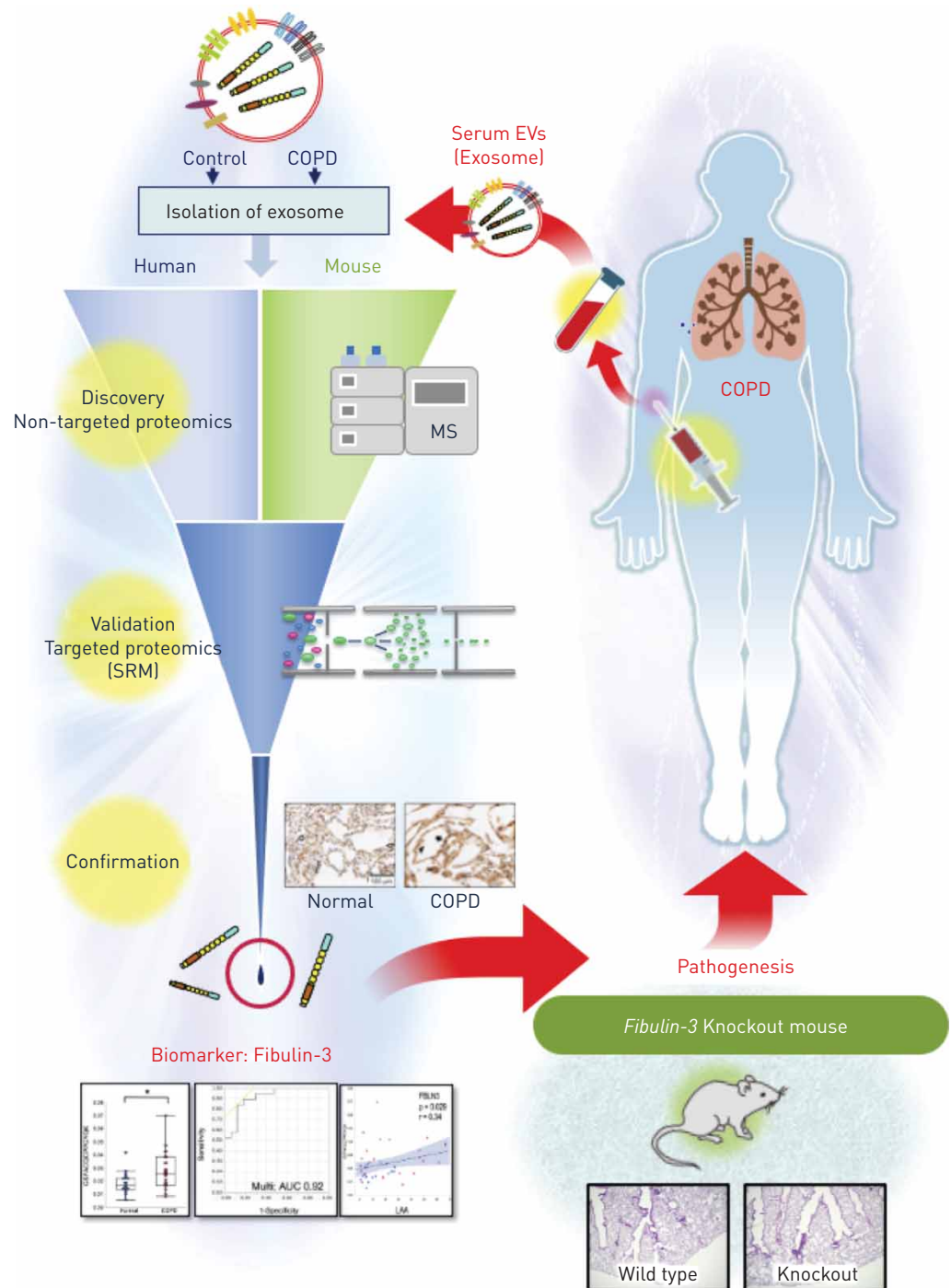


FIGURE 8 A graphical abstract of our study. We identified novel biomarkers using next-generation proteomics for the analyses of serum extracellular vesicles (EVs). In the discovery phase, serum EVs isolated by ultracentrifugation from patients with COPD and healthy controls were analysed. Simultaneously, serum EVs from elastase-treated mouse models were similarly analysed. In the validation phase, biomarker candidates from both humans and mice were further quantified by targeted proteomics, selected reaction monitoring (SRM). Finally, upregulation of fibulin-3 was confirmed by immunoblotting of EVs and immunohistochemistry in lungs. Fibulin-3, a pathogenic matricellular protein in elastic fibres, could be a novel biomarker for COPD. Because fibulin-3 knockout mice spontaneously developed emphysema with age, they could be used to reveal the pathogenesis of COPD. Hence, our strategy could become a novel strategy for biomarker discovery and precision medicine.

genome-wide association studies, *fibulin-3* associated with forced vital capacity [40]. Although *fibulin-3* knockout mice are reported to develop early ageing, herniation, and failure of pelvic organ support, there have been no reports regarding the involvement of fibulin-3 in emphysema in either humans or mice

[27, 28]. Although we could not address its mechanism of emphysema in *fibulin-3* knockout mice, as such a study would have been beyond our scope, the mechanism could be due to the lack of constituent components of elastic fibres. Alternatively, increased MMP-9 might cause emphysema, because lack of fibulin-3 increases MMP-9 activity, thereby inducing pelvic herniation [28]. Further studies are needed to elucidate the function of EVs with fibulin-3.

In addition, other proteins closely connected to lung function, emphysema and COPD were also identified in our discovery phase, which warrants further study alongside fibulin-3. TPP2, a peptidase downstream of the proteasome, was also significantly correlated with lung function and emphysema. Although several studies have demonstrated the importance of proteasomal degradation of modified and misfolded proteins in response to cigarette smoke exposure, none has clarified the role of TPP2 in the pathogenesis of COPD. As *TPP2* heterozygous knockout mice show an ageing phenotype [41], TPP2 could be developed as a target in the COPD pathophysiology.

Tetraspanins, including CD9 and CD81, interact with a wide variety of transmembrane proteins, thereby modulating their functions [42]. We previously found that CD9/CD81 double knockout mice spontaneously develop a COPD-like phenotype, as well as a syndrome resembling human ageing, including cataracts, hair loss, muscle atrophy, and shorter survival [43]. Given that CD81 constitutes a key player in COPD/ageing and EV biology, and that the upregulation of CD81 was associated with emphysema (fig. 5b), further research is warranted to investigate whether CD81 and CD9 might affect both COPD and ageing through modulation of EVs.

While our strategy has a variety of advantages, it does have a few limitations in terms of clinical applications of biomarkers for COPD. The number of participants included in this study was relatively small; thus, COPD endotypes or phenotypes were not characterised in this study, and it is necessary to confirm the repeatability of the results in a large-scale cohort. As EVs are influenced by demographic variations such as sex, age, smoking status, and other factors, we should be prudent about the differences in patient characteristics [19, 44]. Although the people in the COPD group were older compared with those in the healthy group in the validation phase, analysis of covariance confirmed the significant difference in fibulin-3 regardless of age.

Giving that the EV population depends on the isolation method, another limitation of this study was the use of 0.22- μm spin filters before ultracentrifugation. Cells release EVs of different sizes, and their heterogeneity poses major obstacles to our understanding of the composition and functional properties of distinct secreted components [19]. In addition, we used sucrose cushion ultracentrifugation, rather than sucrose density gradient, for the discovery phase. Density gradient centrifugation has the advantage of obtaining EVs with higher purity, but its disadvantage is that it takes more time and energy [45].

Although the strength of fibulin-3 in diagnosing COPD was not very high, the upregulation of fibulin-3 was correlated with lung function and emphysema. However, it was reasonable, considering the nature of COPD defined by forced expiratory volume. Currently, one of the promising COPD biomarkers is an isoform of the transmembrane receptor for advanced glycosylation end products (RAGE). Plasma-soluble RAGE is negatively correlated with emphysema, whereas fibulin-3 and TPP2 are positively correlated with emphysema [46]. Moreover, the correlation values between FEV₁ and fibulin-3 and between FEV₁ and TPP2 ($r=-0.041$ and -0.44 , respectively) were both higher than that of soluble RAGE ($r=0.14$) [47].

Although fibulin-3 alone could be useful for diagnosis and prediction of lung function, multiple biomarkers would be more helpful than individual biomarkers for predicting subtypes, disease severity, and mortality. Moreover, genes, proteins, and metabolite networks are particularly important, as the explanatory value of any single molecule is small compared with multiple biomarkers [8]. These biomarkers, either in multiple biomarker panels or integrated with other omics, may lead to novel diagnostic and prognostic tests for COPD phenotypes [9]. A system-biology platform that stems from advancements in medical diagnosis, omics, and bioinformatics could offer great potential to better understand the complexity of COPD [4].

We identified novel biomarkers using next-generation proteomics for the analyses of serum EVs. Among them, fibulin-3, a pathogenic matricellular protein in elastic fibres, may be a novel biomarker for COPD. Our strategy could be useful for investigations involving system biology and personalised medicine.

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References

- 1 GBD 2015 Chronic Respiratory Disease Collaborators. Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Respir Med* 2017; 5: 691–706.
- 2 Agusti A. The path to personalised medicine in COPD. *Thorax* 2014; 69: 857–864.
- 3 O’Neil SE, Lundback B, Lotvall J. Proteomics in asthma and COPD phenotypes and endotypes for biomarker discovery and improved understanding of disease entities. *J Proteomics* 2011; 75: 192–201.
- 4 Noell G, Faner R, Agusti A. From systems biology to P4 medicine: applications in respiratory medicine. *Eur Respir Rev* 2018; 27: 170110.
- 5 Ito K, Barnes PJ. COPD as a disease of accelerated lung aging. *Chest* 2009; 135: 173–180.
- 6 Stockley RA, Halpin DMG, Celli BR, et al. Chronic obstructive pulmonary disease biomarkers and their interpretation. *Am J Respir Crit Care Med* 2019; 199: 1195–1204.
- 7 Faner R, Tal-Singer R, Riley JH, et al. Lessons from ECLIPSE: a review of COPD biomarkers. *Thorax* 2014; 69: 666–672.
- 8 Regan EA, Hersh CP, Castaldi PJ, et al. Omics and the search for blood biomarkers in chronic obstructive pulmonary disease. Insights from COPDGene. *Am J Respir Cell Mol Biol* 2019; 61: 143–149.
- 9 Liu Z, Li W, Lv J, et al. Identification of potential COPD genes based on multi-omics data at the functional level. *Mol Biosyst* 2016; 12: 191–204.
- 10 Shendure J, Balasubramanian S, Church GM, et al. DNA sequencing at 40: past, present and future. *Nature* 2017; 550: 345–353.
- 11 Bartel DP. Metazoan MicroRNAs. *Cell* 2018; 173: 20–51.
- 12 Aebersold R, Mann M. Mass-spectrometric exploration of proteome structure and function. *Nature* 2016; 537: 347–355.
- 13 Verrills NM, Irwin JA, He XY, et al. Identification of novel diagnostic biomarkers for asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2011; 183: 1633–1643.
- 14 Shiromizu T, Kume H, Ishida M, et al. Quantitation of putative colorectal cancer biomarker candidates in serum extracellular vesicles by targeted proteomics. *Sci Rep* 2017; 7: 12782.
- 15 Narumi R, Murakami T, Kuga T, et al. A strategy for large-scale phosphoproteomics and SRM-based validation of human breast cancer tissue samples. *J Proteome Res* 2012; 11: 5311–5322.
- 16 Narumi R, Shimizu Y, Ukai-Tadenuma M, et al. Mass spectrometry-based absolute quantification reveals rhythmic variation of mouse circadian clock proteins. *Proc Natl Acad Sci USA* 2016; 113: E3461–E3467.
- 17 Tirumalai RS, Chan KC, Prieto DA, et al. Characterization of the low molecular weight human serum proteome. *Mol Cell Proteomics* 2003; 2: 1096–1103.
- 18 Tkach M, Thery C. Communication by extracellular vesicles: where we are and where we need to go. *Cell* 2016; 164: 1226–1232.
- 19 Thery C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018; 7: 1535750.
- 20 Hoshino A, Costa-Silva B, Shen TL, et al. Tumour exosome integrins determine organotropic metastasis. *Nature* 2015; 527: 329–335.
- 21 Ueda K, Ishikawa N, Tatsuguchi A, et al. Antibody-coupled monolithic silica microtips for high-throughput molecular profiling of circulating exosomes. *Sci Rep* 2014; 4: 6232.
- 22 Genschmer KR, Russell DW, Lal C, et al. Activated PMN exosomes: pathogenic entities causing matrix destruction and disease in the lung. *Cell* 2019; 176: 113–126.e115.
- 23 Conickx G, Mestdagh P, Avila Cobos F, et al. MicroRNA profiling reveals a role for microRNA-218-5p in the pathogenesis of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2017; 195: 43–56.
- 24 Tetsumoto S, Takeda Y, Imai H, et al. Validation of noninvasive morphological and diffusion imaging in mouse emphysema by micro-computed tomography and hyperpolarized (¹²⁹Xe) magnetic resonance imaging. *Am J Respir Cell Mol Biol* 2013; 49: 592–600.
- 25 Yang L, Broom MF, Tucker IG. Characterization of a nanoparticulate drug delivery system using scanning ion occlusion sensing. *Pharm Res* 2012; 29: 2578–2586.
- 26 MacLean B, Tomazela DM, Shulman N, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 2010; 26: 966–968.
- 27 McLaughlin PJ, Bakall B, Choi J, et al. Lack of fibulin-3 causes early aging and herniation, but not macular degeneration in mice. *Hum Mol Genet* 2007; 16: 3059–3070.
- 28 Rahn DD, Acevedo JF, Roshanravan S, et al. Failure of pelvic organ support in mice deficient in fibulin-3. *Am J Pathol* 2009; 174: 206–215.
- 29 Melo SA, Luecke LB, Kahlert C, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 2015; 523: 177–182.
- 30 Haenen S, Clynen E, Nemery B, et al. Biomarker discovery in asthma and COPD: application of proteomics techniques in human and mice. *EuPA Open Proteomics* 2014; 4: 101–112.
- 31 Luisetti M, Ma S, Iadarola P, et al. Desmosine as a biomarker of elastin degradation in COPD: current status and future directions. *Eur Respir J* 2008; 32: 1146–1157.
- 32 Nakamura T, Lozano PR, Ikeda Y, et al. Fibulin-5/DANCE is essential for elastogenesis *in vivo*. *Nature* 2002; 415: 171–175.
- 33 Liu G, Cooley MA, Jarnicki AG, et al. Fibulin-1 regulates the pathogenesis of tissue remodeling in respiratory diseases. *JCI Insight* 2016; 1: e86380.

- 34 Nakamura T. Roles of short fibulins, a family of matricellular proteins, in lung matrix assembly and disease. *Matrix Biol* 2018; 73: 21–33.
- 35 Brandsma CA, van den Berge M, Postma DS, et al. A large lung gene expression study identifying fibulin-5 as a novel player in tissue repair in COPD. *Thorax* 2015; 70: 21–32.
- 36 Kuang PP, Joyce-Brady M, Zhang XH, et al. Fibulin-5 gene expression in human lung fibroblasts is regulated by TGF-beta and phosphatidylinositol 3-kinase activity. *Am J Physiol Cell Physiol* 2006; 291: C1412–C1421.
- 37 Hirai M, Ohbayashi T, Horiguchi M, et al. Fibulin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage in vivo. *J Cell Biol* 2007; 176: 1061–1071.
- 38 Nakanishi K, Takeda Y, Tetsumoto S, et al. Involvement of endothelial apoptosis underlying chronic obstructive pulmonary disease-like phenotype in adiponectin-null mice: implications for therapy. *Am J Respir Crit Care Med* 2011; 183: 1164–1175.
- 39 Yoshida M, Whitsett JA. Alveolar macrophages and emphysema in surfactant protein-D-deficient mice. *Respirology* 2006; 11 Suppl: S37–S40.
- 40 Loth DW, Soler Artigas M, Gharib SA, et al. Genome-wide association analysis identifies six new loci associated with forced vital capacity. *Nat Genet* 2014; 46: 669–677.
- 41 McKay RM, McKay JP, Suh JM, et al. Tripeptidyl peptidase II promotes fat formation in a conserved fashion. *EMBO Rep* 2007; 8: 1183–1189.
- 42 Hemler ME. Tetraspanin proteins promote multiple cancer stages. *Nat Rev Cancer* 2014; 14: 49–60.
- 43 Jin Y, Takeda Y, Kondo Y, et al. Double deletion of tetraspanins CD9 and CD81 in mice leads to a syndrome resembling accelerated aging. *Sci Rep* 2018; 8: 5145.
- 44 Baek R, Varming K, Jorgensen MM. Does smoking, age or gender affect the protein phenotype of extracellular vesicles in plasma? *Transfus Apher Sci* 2016; 55: 44–52.
- 45 Bobrie A, Colombo M, Krumeich S, et al. Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. *J Extracell Vesicles* 2012; 1: 18397.
- 46 Casaburi R, Celli B, Crapo J, et al. The COPD Biomarker Qualification Consortium (CBQC). *COPD* 2013; 10: 367–377.
- 47 Iwamoto H, Gao J, Pulkkinen V, et al. Soluble receptor for advanced glycation end-products and progression of airway disease. *BMC Pulm Med* 2014; 14: 68.