Alternative Splicing Is a Major Factor Shaping Transcriptome Diversity in Mild and Severe COPD

Khalenkow, Dmitry; Brandsma, Corry-Anke; Timens, Wim; Choy, David F; Grimbaldeston, Michele A; Rosenberger, Carrie M; Slebos, Dirk-Jan; Kerstjens, Huib A M; Faiz, Alen; Koppelman, Gerard H

Published in:
American Journal of Respiratory Cell and Molecular Biology

DOI:
10.1165/rcmb.2023-0296OC

ORIGINAL RESEARCH

Alternative Splicing Is a Major Factor Shaping Transcriptome Diversity in Mild and Severe Chronic Obstructive Pulmonary Disease

Dmitry Khalenkow[1,2,3], Corry-Anke Brandsma[2,4], Wim Timens[2,4], David F. Choy[7], Michele A. Grimbaldeston[7], Carrie M. Rosenberger[7], Dirk-Jan Slebos[5], Huib A. M. Kerstjens[6], Alen Faiz[8], Gerard H. Koppelman[2,3], Martijn C. Nawijn[2,4], Maarten van den Berge[2,5*], and Victor Guryev[1*]

[1]European Research Institute for the Biology of Ageing, [2]Groningen Research Institute for Asthma and COPD, [3]Department of Pediatric Pulmonology and Pediatric Allergology, Beatrix Children's Hospital, [4]Department of Pathology and Medical Biology, [5]Department of Pulmonary Diseases, and [6]Department of Pulmonology and Tuberculosis, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; [7]Genentech, Inc., South San Francisco, California; and [8]Faculty of Science, Centre for Inflammation, Centenary Institute and University of Technology Sydney, Sydney, Australia

ORCID IDs: 0000-0001-6786-7774 (D.K.); 0000-0001-8911-3658 (C.-A.B.); 0000-0002-4146-6363 (W.T.); 0000-0003-1351-6113 (D.F.C.); 0000-0001-6372-8099 (M.A.G.); 0000-0002-8189-8822 (C.M.R.); 0000-0001-9555-3422 (D.-J.S.); 0000-0001-7705-7927 (H.A.M.K.); 0000-0003-1740-3538 (A.F.); 0000-0001-8567-3252 (G.H.K.); 0000-0003-3372-6521 (M.C.N.); 0000-0002-9336-7340 (M.v.d.B.); 0000-0002-5810-6022 (V.G.).

Abstract

The role of alternative splicing in chronic obstructive pulmonary disease (COPD) is still largely unknown. We aimed to investigate the differences in alternatively splicing events between patients with mild-to-moderate and severe COPD compared with non-COPD control subjects and to identify splicing factors associated with aberrant alternative splicing in COPD. For this purpose, we performed genome-wide RNA-sequencing analysis of bronchial brushings from 23 patients with mild-to-moderate COPD, 121 with severe COPD, and 23 non-COPD control subjects. We found a significant difference in the frequency of alternative splicing events in patients with mild-to-moderate and severe COPD compared with non-COPD control subjects. There were from two to eight times (depending on event type) more differential alternative splicing events in the severe than in the mild-to-moderate stage. The severe COPD samples showed less intron retention and more exon skipping. It is interesting that the transcript levels of the top 10 differentially expressed splicing factors were significantly correlated with the percentage of many alternatively spliced transcripts in severe COPD. The aberrant alternative splicing in severe COPD was predicted to increase the overall protein-coding capacity of gene products. In conclusion, we observed large and significant differences in alternative splicing between bronchial samples of patients with COPD and control subjects, with more events observed in severe than in mild-to-moderate COPD. The changes in the expression of several splicing factors correlated with prevalence of alternative splicing in severe COPD. Alternative splicing can indirectly impact gene expression by changing the relative abundance of protein-coding isoforms potentially influencing pathophysiological changes. The results provide a better understanding of COPD-related alternative splicing changes.

Keywords: sequence analysis; RNA; RNA splicing; lung diseases

Chronic obstructive pulmonary disease (COPD) is characterized by an irreversible and progressive airflow limitation causing respiratory symptoms. COPD is driven by genetic, epigenetic, and environmental factors that may affect gene expression in the lungs (1). Several studies have shown COPD-associated differences in lung transcriptomes (2, 3). Alternatively, spliced isoforms may play a significant role in COPD, as they give rise to unique COPD-specific transcript and protein isoforms (4, 5). Alternative splicing (AS) is a molecular mechanism that allows cells to produce a variety of mRNAs by selecting different combinations of exons within a single pre-mRNA (6). As a result, AS gives cells the ability to produce multiple protein isoforms

(Received in original form August 10, 2023; accepted in final form February 5, 2024)

*Co–senior authors.

This collaboration project is co-financed by Genentech and the Dutch Government (Health Holland) by means of the PPP-allowance to stimulate public-private partnerships

Author Contributions: D.K.: data analysis, interpretation, and manuscript writing. C.-A.B. and W.T.: study design, project support, interpretation, and manuscript writing. D.F.C., M.A.G., and C.M.R.: RNA-sequencing data generation, project support, and manuscript review. D.-J.S.: patient recruitment, sampling, and manuscript editing. H.A.M.K.: study design, patient recruitment, data interpretation and manuscript editing. A.F.: data interpretation and manuscript editing. G.H.K., M.C.N., M.v.d.B., and V.G.: study design, data analysis, interpretation, and manuscript writing.

Correspondence and requests for reprints should be addressed to Victor Guryev, Antonius Deusinglaan 1, UMCG, int zip FA50, Groningen 9713AV, the Netherlands. E-mail: v.guryev@umcg.nl.

A data supplement for this article is available via the Supplements tab at the top of the online article.

Am J Respir Cell Mol Biol Vol 70, Iss 5, pp 414–423, May, 2024

Originally Published in Press as DOI: 10.1165/rcmb.2023-0296OC on February 5, 2024
Internet address: www.atsjournals.org

from the same gene that vary in function and activity. There are five common types of AS events: use of alternative 3′ and 5′ splice sites, mutually exclusive exons, exon skipping, and intron retention (Figure 1). At least 92% of human genes can be alternatively spliced (7). AS may also lead to dysfunctional protein isoforms. Alterations in AS were associated with cancer (8) and with neurological (9, 10), autoimmune (11), and lung (12, 13) diseases. For instance, AS results in a switch between *BCL-X* isoforms that differentially inhibit apoptosis in lung cancer cells (14). Also, exon skipping in the *MET* mRNA transcript results in more stable *MET* protein and significant *in vivo* tumor growth (15). Finally, abnormal exon skipping in the *CFTR* gene can cause or exacerbate cystic fibrosis (16). As such, abnormal expression of splicing factors that regulate AS can contribute to disease development. For instance, increased expression of the splicing factor *SRSF6* has been linked to the development of pleural fibrosis (17). Peripheral blood analysis revealed SNPs in COPD-associated genes influencing AS, suggesting that analysis of AS can provide novel insights into COPD mechanisms (13). However, the role of AS in obstructive airway disease—in particular, COPD—is not known.

Therefore, in this study, we investigated the differences in AS events between patients with mild-to-moderate COPD (Global Initiative for Chronic Obstructive Lung Disease [GOLD 1 and GOLD 2 stages), severe COPD (GOLD 3 and GOLD 4 stages), and non-COPD control subjects. We used RNA-sequencing (RNA-seq) data of bronchial brushings from 23 patients with mild-to-moderate COPD and 121 patients with severe COPD versus 23 non-COPD control individuals to determine whether AS differs between individuals with and those without COPD and whether there is a link between the severity of COPD and changes in AS. In addition, we aimed to establish the link between the differential expression of splicing factors and changes in AS to identify splicing factors that may play a key role in observed differences in AS in COPD.

Methods

Patients and Study Design

The SHERLOCk cohort (an integrative genomic approach to Solve tHe puzzle of sevERe earLy-Onset COPD; ClinicalTrials.gov: NCT04263961) is a cross-sectional observational study without pharmacological intervention. Participants had at least 5 pack-years of smoking, did not smoke for at least 6 months before inclusion, and did not have ever asthma or acute infection in the past 3 months. Subjects underwent bronchoscopy under conscious sedation or general anesthesia, during which bronchial brushings were obtained. The study was approved by the institutional review board, medical ethics committee of UMC Groningen, with written informed consent from all subjects. RNA from bronchial brushes was isolated using the QIAGEN AllPrep DNA/RNA/miRNA Universal Mini Kit according to the manufacturer's instructions. RNA-seq libraries were prepared using the Illumina TruSeq Stranded Total RNA method. The RiboZero Magnetic Gold Kit was used to deplete rRNA from the samples. Libraries were paired-end sequenced. RNA-seq libraries were sequenced in 2 × 51-bp paired-end mode with an average coverage of 55.2 million read pairs per library. Control and COPD samples were processed in the same batch, using the same equipment and protocols. (For information about subsequent RNA-seq data preprocessing, *see* the data supplement.) The RNA-seq data can be accessed from the European Genome-phenome Archive (accession number pending).

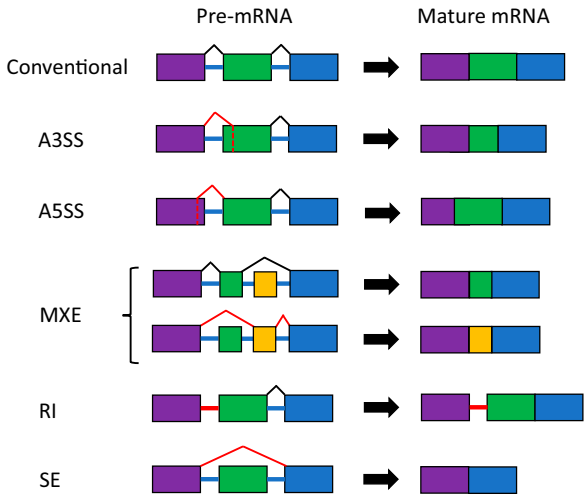

Figure 1. Schematic representation of different types of alternative splicing events. A3SS = alternative 3′-splice site; A5SS = alternative 5′-splice site; Conventional = conventional splicing; MXE = mutually exclusive exon; RI = retained intron; SE = skipped exon.

AS Analysis

Patients at GOLD 1 and GOLD 2 stages were combined into a mild-to-moderate COPD group, whereas those at GOLD 3 and GOLD 4 stages were grouped as severe COPD. The control group consisted of participants without COPD. The rMATS-turbo (v.4.1.0.) program was used for the detection of differential AS events by groupwise comparison using a likelihood-ratio test (18). The mild-to-moderate and severe COPD transcriptomes were compared with those of the control samples. Additionally, the severe COPD group was compared with the mild-to-moderate group. The analysis of the resulting datasets was conducted in R, Version 4.0.3. The Benjamini–Hochberg false discovery rate (FDR) <0.05 was used to filter the genes with significant differences in the frequency of AS events for each category of AS events. The inclusion level difference from rMATS output was used to determine whether the change in AS resulted in an increase or decrease in the proportion of transcripts exhibiting a specific splicing event for a particular gene of interest. Information about permutation analysis, which was conducted to investigate the effect of imbalance in group sizes between non-COPD subjects and patients with severe COPD on AS is provided elsewhere (*see* the data supplement).

Identification of Splicing Factors Correlated with Differences in AS in Severe COPD

For each splicing factor gene, differentially expressed in severe COPD, we investigated the number of AS events, where the differences in "percent spliced in" (the ratio of the relative abundance of isoforms containing a certain exon of interest over the relative

abundance of all isoforms of the gene) from rMATS output across all samples significantly correlates (FDR, <0.05) with differences in expression of this splicing factor in corresponding samples in transcriptome data. (For details on the methods used in this study, *see* the data supplement.)

Results

The baseline clinical characteristics of the study groups are summarized in Table 1.

Different Patterns of AS in Patients with Mild-to-Moderate and Severe COPD

We compared the per-gene frequency of AS events in transcriptomic data of bronchial brushings from patients with mild-to-moderate and severe COPD, relative to control samples. The total number of genes for which the frequency of AS events differs between mild-to-moderate or severe COPD and control (differential AS events) and that pass the significance threshold (FDR, <0.05) is shown in Figure 2A. We observed more intron retention than exon-skipping events in patients with mild-to-moderate COPD (Figures 2A, 2E, and 2F). In general, the composition of AS event types exhibits remarkable differences when comparing mild-to-moderate with severe COPD groups (Figures 2B–2F). Thus, we see exon skipping as more prominent in patients with severe COPD, in contrast to those with mild-to-moderate COPD (Figures 2A and 2F). Specifically for patients with severe COPD, we have detected a lower prevalence of AS events with retained introns and a higher number of AS events with exon skipping. It is interesting to note that five times more differential AS events were observed with an increased percentage of transcripts exhibiting skipped exons in patients with severe COPD than in those with mild-to-moderate COPD when each of them was compared with control subjects (Figures 2E and 2F). At the same time, up to 10 times more differential AS events with a lower percentage of transcripts with retained introns were observed in patients with severe COPD than in patients with mild-to-moderate COPD when each group was compared with control subjects, (Figures 2D and 2F). It should be noted that, because of the difference in group sizes, patients with severe COPD were overrepresented in our analysis. In light of the larger size of our patient group with severe COPD compared with others, we conducted a permutation analysis on a balanced subset to ensure that the observed differences in AS were not merely due to group size disparities (*see* Figure E1 in the data supplement). The results of this analysis were in line with observations reported for the full dataset. We also directly compared severe COPD and mild-to-moderate groups and identified the subset of differential AS events where the frequency of AS events differs between severe and mild-to-moderate COPD (*see* Figure E2). It is interesting to note that these differential AS events only partially overlap with events identified by comparison of severe COPD with control (*see* Figure E3).

The Limited Overlap across AS Event Type and COPD Severity

Next, the genes affected by AS in mild-to-moderate and severe COPD datasets were cross-compared to evaluate how many of those genes are COPD stage–specific and how many affected genes overlap between these datasets. Less than half of the genes with a significantly changed frequency of mutually exclusive exons, retained introns, or skipped exons, present in the mild-to-moderate COPD dataset, were also found in severe COPD (Figures 3A and 3B). Alternative 3′-splice site and alternative 5′-splice site AS events had the least overlap between the datasets. It is interesting to note that we found only a few genes in severe COPD where different types of AS events were present in the same gene (*see* Figure E4). Most of the genes, where differences in AS event frequency were detected, exhibit only one type of AS event per transcribed gene.

Protein-Coding Isoforms Increase in Patients with Severe COPD versus Control Subjects

We analyzed the predicted effect of differential AS on the protein-coding capacity of the transcriptome. Without full-length long-read data and/or proteomic data, it is impossible to unambiguously assign each AS exon combination to a coding or a noncoding isoform. Thus, we used an alternative approach: distinguishing AS exon combinations compatible with one or more protein-coding transcripts (potentially coding) from those that are incompatible with any protein-coding isoforms (noncoding). Although this definition does not guarantee the functional discordance between alternatively spliced isoforms, it should enrich for events where AS alters isoform coding capacity. To this end, we focused on the cases when the transcripts carrying the AS junction were not annotated as protein coding, whereas one of the reference transcripts was (or vice versa). It was found that the majority of transcripts that resulted from differential AS events

Table 1. Baseline Clinical Characteristics of the Study Groups

Characteristic	Control	Mild-to-Moderate (GOLD 1–2)	Severe (GOLD 3–4)
Group size, *n*	23	23	121
Age, median (IQR)	60 (52–63)	62 (57–65)	60 (56–64)
Male/female, *n*	12/11	18/5	34/87
Pack-years, median (IQR)	28 (14–48)	56 (23–74)	36 (30–46)
Current/ex-smokers, *n*	0/23	0/23	1/120
FEV_1, % predicted, median (IQR)	102 (90–110)	77 (68–89)	24 (20–29)
FEV_1/FVC, ratio median (IQR)	0.73 (0.70–0.77)	0.55 (0.5–0.59)	0.28 (0.24–0.32)
ICS before inclusion, *n* (%)	0 (0)	11 (48)	108 (89)
%LAA, median (IQR)	—	—	42 (38–46)*

Definition of abbreviations: GOLD = Global Initiative for Chronic Obstructive Lung Disease; ICS = inhaled corticoid steroid; IQR = interquartile range in male, female. %LAA = percentage of low attenuation area at a −950 HU threshold.
*Based on computed tomography scan of 101 patients.

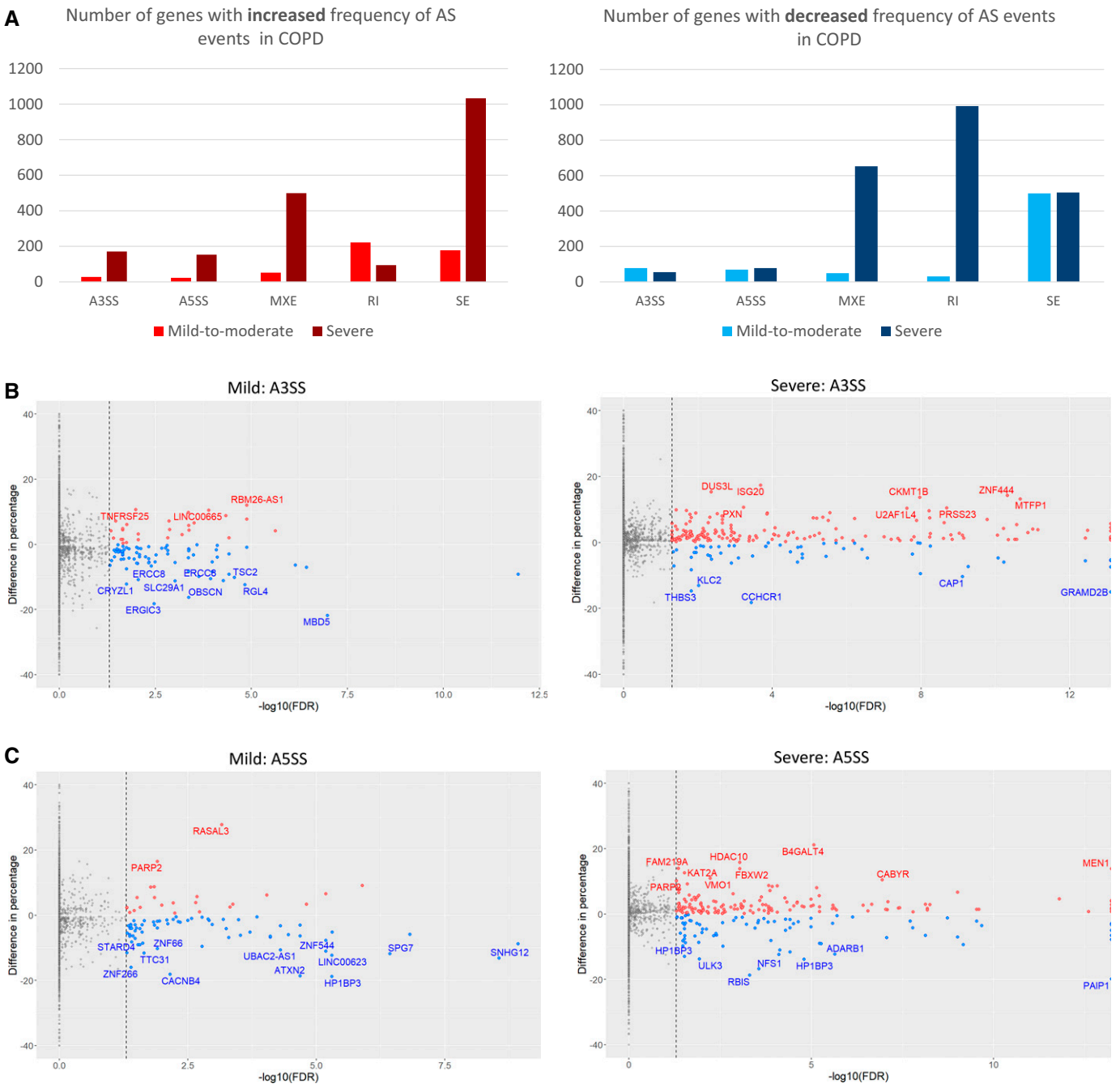

Figure 2. Alternative splicing (AS) in mild-to-moderate and severe chronic obstructive pulmonary disease (COPD). (*A*) Total number of genes with significantly (left) increased or (right) decreased frequency of alternative splicing events in mild-to-moderate and severe COPD. (*B–F*) The difference in the percentage of corresponding alternative exons inclusion levels in patients with mild-to-moderate or severe COPD relative to control subjects. Red dots correspond to significant differential AS events (false discovery rate [FDR], <0.05) with increased inclusion levels; blue dots correspond to those with decreased inclusion levels. (*B*) Differential AS event with alternative 3′-(acceptor) splice site (A3SS; red corresponds to an increase in usage of a shorter and blue of a longer exon in COPD samples). (*C*) Differential AS event with alternative 5′-(donor) splice site (A5SS; similar to A3SS). (*D*) Differential AS event with MXE. Red corresponds to an increase in the inclusion of downstream cassette exons and blue, to an increase in the inclusion of upstream cassette exons, in COPD samples. (*E*) Differential AS event with RI. Red corresponds to more frequent intron excision and blue, to intron retention, in COPD samples. (*F*) Differential AS event with SE. Red corresponds to more frequent exon skipping and blue, to exon inclusion, in patients with COPD.

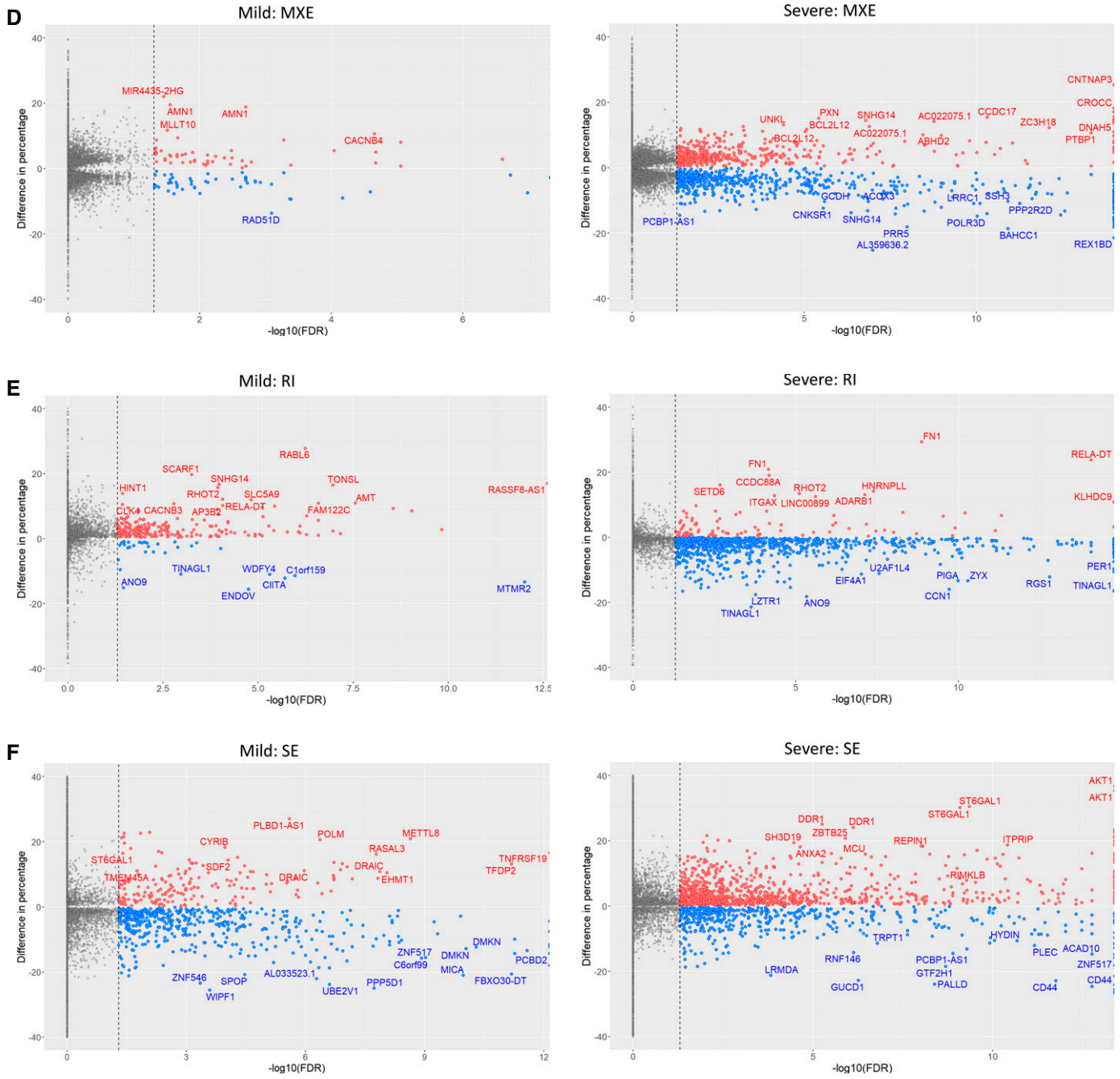

Figure 2. (*Continued*).

and were discordant in their protein-coding capacity are protein coding in patients with severe COPD, whereas the noncoding isoforms show increased presence in control subjects (Figure 4). Next, we identified genes with a relative difference in isoform frequency that was more than 10% in patients with severe COPD compared with control subjects and where the differential AS event may disrupt the protein-coding capacity of the gene. *Fibronectin 1* (*FN1*) and *Cellular Communication Network Factor 1* (*CCN1*) were among the top genes found in our analysis. *FN1* had a 20% increase in non–protein-coding transcripts with intron retention in severe COPD, and its gene expression was downregulated. *CCN1* had a 16% decrease in non–protein-coding transcripts with a retained intron in severe COPD, and its gene expression was downregulated as well. (For the resulting full gene list, *see* Table E1. For the sashimi plots of AS events in *FN1* and *CCN1* genes, *see* Figure E5.)

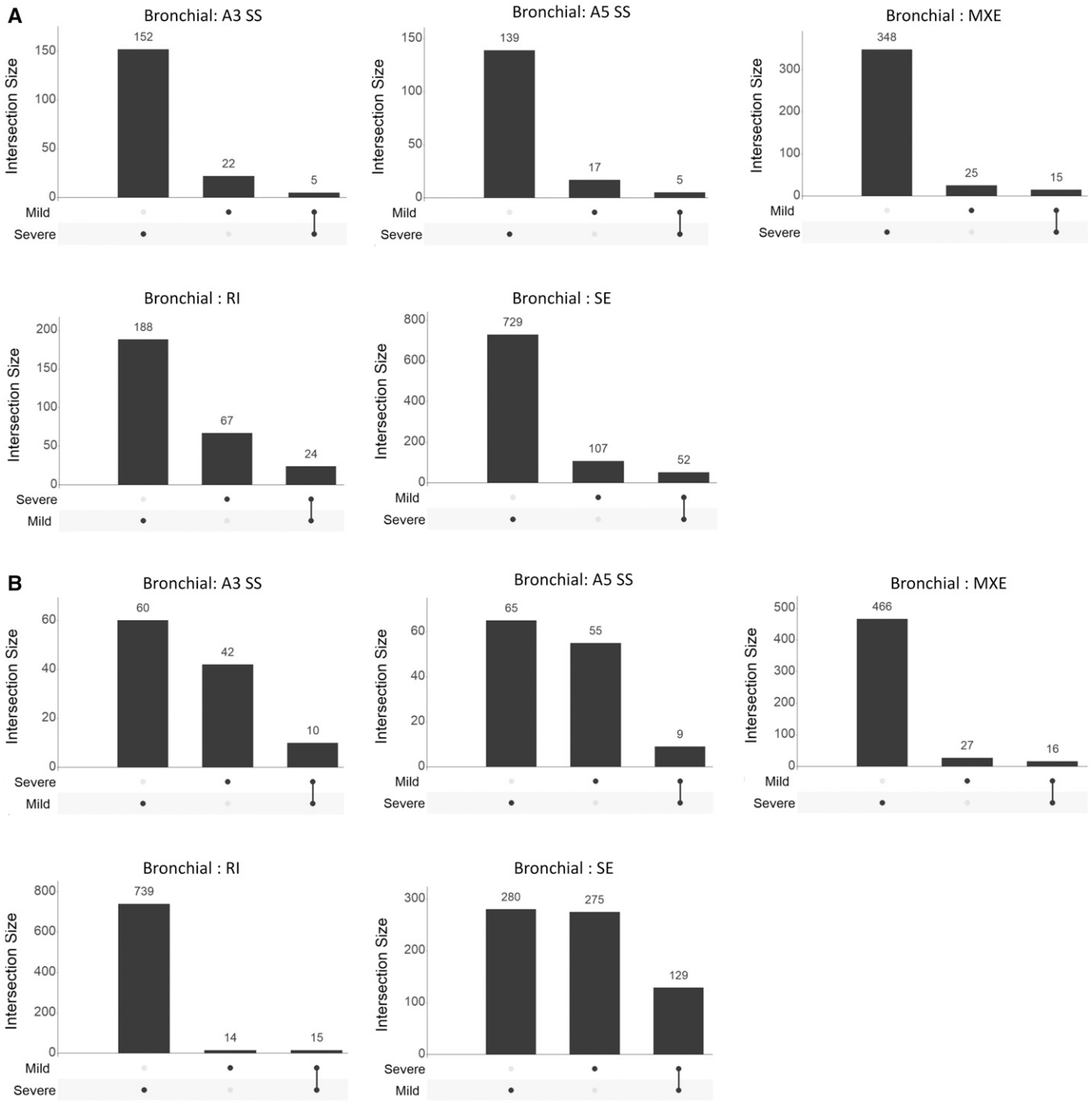

Figure 3. Comparison of subsets of genes expressed in bronchial brushes with (*A*) increased or (*B*) decreased frequency of AS events compared with control. Mild indicates genes with changed frequency of AS events in samples of mild-to-moderate COPD. Severe indicates genes with changed frequency of AS events in severe COPD samples. The bar with a single dot indicates the number of unique genes found only in this dataset. Bars marked by dots with lines represent the overlaps between the corresponding subsets.

Differential Expression of Splicing Factors Is Associated with Splicing Changes in Patients with COPD

Finally, we investigated whether the differential expression of one or more splicing factors was correlated with the observed changes in splicing. We detected expression in bronchial samples of a total of 327 genes encoding proteins annotated in UniProt as splicing factors or RNA-associated proteins. Next, we tested the association of the expression of each of these genes with the relative frequency of transcripts carrying an AS event in each disease condition. Per type of AS event, the

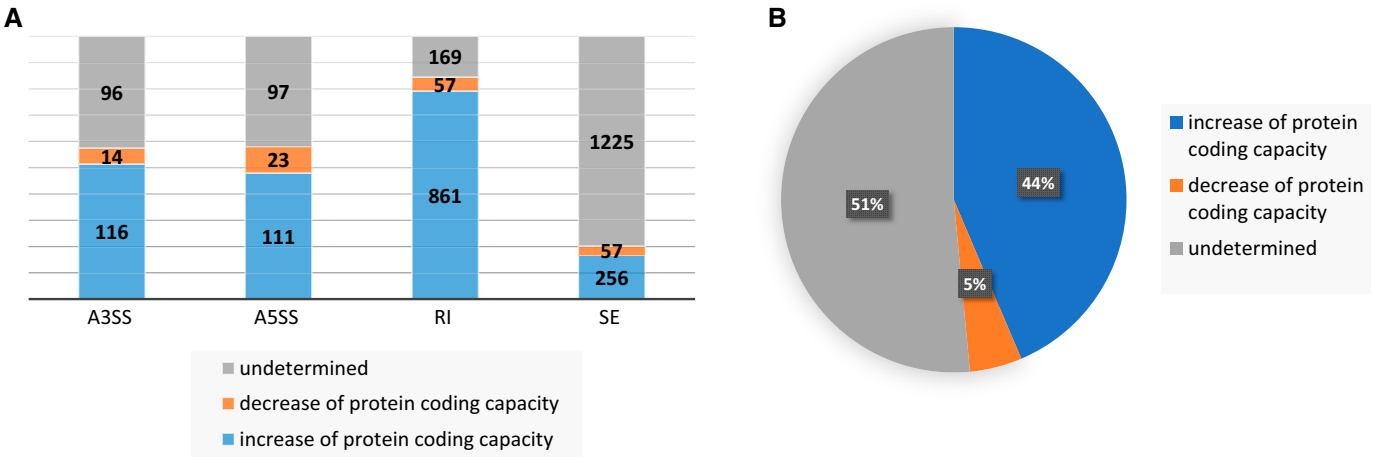

Figure 4. The impact of differential AS events on the protein-coding capacity of genes affected by alternative splicing in severe COPD. (*A*) The number of significant differential events (FDR, <0.05) for each category of AS events that resulted in an increase or decrease of protein-coding isoforms in patients with severe COPD compared with that in control subjects. (*B*) The total percentage of AS events that resulted in an increase, or decrease, of protein-coding capacity. Differential AS events, for which it was not possible to evaluate the effect on protein-coding capacity, were categorized as undetermined.

expression of the 10 most significant splicing factor genes in bronchial samples of patients with severe COPD was correlated with changes in frequencies of significant AS events. The percentage of observed prominent correlations ($R^2 > 0.5$) between the expression of these splicing factor genes and the frequency of AS events are summarized in Table 2.

Discussion

To our knowledge, this is the first study that systematically investigates AS in bronchial brushings of a large number of patients with COPD. We observed marked differences in the frequency of AS events between patients with mild-to-moderate and severe COPD when compared with matched control subjects. We showed that COPD severity has a strong association with the directionality and number of AS events. We detected from two to four times (depending on AS event type) more differential AS events in severe COPD than in mild-to-moderate COPD (Figure 2F). In addition, our findings suggest that subsets of differential AS events are highly enriched in a COPD stage–specific

Table 2. Top 10 Spliced Factors Differentially Expressed in Severe COPD and Positively Correlated (Correlation Factor, >0.5) with Changes in AS Isoform Proportions in Bronchial Tissue Samples

	Percentage of AS Events Correlated with Expression of Splicing Factor				Splicing Factor Differential Expression in Severe COPD versus Control		
Gene Symbol	A3SS	A5SS	RI	SE	logFC	FDR	Description
FUS	28.3	22.5	51.5	18.5	−0.229	0.01289	FUS RNA-binding protein
RBM5	27	22.1	49.2	14.5	−0.144	0.01204	RNA-binding motif protein 5
DDX17	25.2	21.2	46.9	13.2	−0.132	0.00663	DEAD-box helicase 17
LUC7L3	23.9	20.3	42	12	−0.213	0.00034	LUC7-like 3 pre-mRNA splicing factor
CPSF7	22.1	16.9	38.9	10.8	−0.083	0.01266	Cleavage and polyadenylation–specific factor 7
HNRNPH1	20.4	19.5	43.6	12.4	−0.173	0.00603	Heterogeneous nuclear ribonucleoprotein H1
RBM25	18.1	16	34.7	9.1	−0.126	0.00654	RNA-binding motif protein 25
LUC7L2	17.3	13.9	33.9	8.5	−0.151	0.01144	LUC7-like 2, pre-mRNA splicing factor
RBM39	14.6	14.3	33.6	9.1	−0.148	0.00005	RNA-binding motif protein 39
RNPC3	13.3	13.9	28.3	7	−0.191	0.02335	RNA-binding region (RNP1, RRM) containing 3

Definition of abbreviations: A3SS = alternative 3′-splice site, A5SS = alternative 3′-splice site; AS = alternative splicing; COPD = chronic obstructive pulmonary disease; FDR = false discovery rate; logFC = $\log_2$ fold change; RI = intron retention; SE = skipped exon.
The gene symbol column contains the list of splicing factors. Numbers in the columns of corresponding AS event types (A3SS, A5SS, RI, and SE) indicate the percentage of total differential AS events in severe COPD, which positively correlates with this particular splicing factor. logFC and FDR indicate the $\log_2$ fold change in gene expression and FDR of corresponding splice factor in patients with severe COPD relative to control subjects.

manner, as there was only a partial overlap between the differential AS events in severe and mild-to-moderate COPD (Figures 3 and E2 and E3). We hypothesized that patients with severe COPD and extensive emphysema constitute a distinct clinical phenotype with potentially divergent pathogenesis. The observed dissimilarities in AS patterns between severe COPD and mild-to-moderate COPD, along with the limited overlap, further support this notion.

We detected a decrease in intron retention and an increase in exon skipping in patients with severe COPD compared with control subjects. The detection of a large number of differential AS events with decreased intron retention in patients with severe COPD relative to control subjects did not meet our initial expectations. COPD-related molecular signatures were associated with accelerated aging (19, 20), whereas aging as such is a process that was previously correlated with an increase in intron retention events (21).

By analyzing the correlation between differentially expressed splicing factors and AS events, we identified splicing factors that are highly correlated with a subset of differential AS events. We speculate that changes in the expression of those splicing factors may be the cause of part of the changes in AS in severe COPD. Among the identified factors (Table 2), the *heterogeneous nuclear ribonucleoprotein H1* (*HNRNPH1*) is particularly interesting. The expression of this gene was found to be highly correlated with all types of AS events in the bronchial dataset and was downregulated in patients with severe COPD. *HNRNPH1* is known to modulate AS where, depending on the position of its binding site, it can either promote or downregulate the mRNA splicing (22). Recently, it was discovered that *HNRNPH1* cotranscriptionally represses splicing in macrophages (23). It was also found that stress conditions such as hypoxia led to an increase of cytosolic *HNRNPH1* binding to mRNA (24) and that this gene undergoes hypermethylation in smokers (25). Therefore, the observed connection between COPD and *HNRNPH1* is particularly interesting for further investigation.

Of note, we have found that different types of AS events (retained intron, skipped exon, alternative 3′-splice site, and alternative 5′-splice site) were positively correlated with the expression of a common set of splicing factors. However, there was only a minor difference in gene expression of this subset of splicing factors between patients with severe COPD and control subjects, demonstrating the possibility that even a small change in SF gene expression may have a substantial effect on AS.

The important question is how changes in AS affect cell function. Although it was found that AS causes a shift to more protein-coding isoforms of AS-affected genes in severe COPD relative to control, the majority of detected differential AS events resulted in a difference less than 5% in isoform relative proportions. Therefore, it is challenging to determine whether such a small change would cause a significant change in cellular functions. In our study, we selected the differential AS events with a difference more than 10% between control and severe COPD and focused on cases where AS may cause the disturbance of the protein-coding capacity of the corresponding genes. It is interesting to note that the majority of such differential AS events were found in severe COPD in non–differentially expressed genes or genes with a log fold change of less than 0.5. It is important to note that COPD is a disease that develops over time, and small changes in gene expression or AS events may accumulate and may have a significant impact on disease progression. Therefore, even minor changes in gene expression or AS that may seem insignificant at first might have a profound effect on the development and severity of COPD over time.

Our results might identify novel genes that contribute to COPD pathogenesis through AS events, as these cannot be detected using conventional differential gene expression analysis. Particularly, disease-specific AS patterns may result in more or less protein-coding mRNA transcripts, effectively "upregulating" or "downregulating" the protein levels, whereas the total mRNA expression could be similar between COPD and control. However, one should also consider the possibility that large changes in AS (e.g., skipping of a large exon) may also inflate or decrease the total amount of gene reads. Further, our pathway enrichment analysis did not identify significant pathways among the genes affected by AS (data not shown). We conclude that COPD-related splicing changes involve many genes that are not confined to specific biological pathways but reflect a generic process that is potentially related to the pathogenesis of COPD.

One of the genes affected by this phenomenon was *FN1*, a known extracellular matrix gene that is associated with several pathological processes in the pulmonary system associated with aberrant tissue repair, such as fibrosis (26) or COPD (27). We found a 20% increase of *FN1* non–protein-coding isoform that was due to intron retention in severe COPD relative to control. Next to that, the differential gene expression (DGE) analysis also showed a significant downregulation of this gene expression in severe COPD. This decrease in gene expression may be further amplified by an increase in the production of non–protein-coding AS isoforms, potentially translating to an even stronger decrease of *FN1* gene product on protein level.

For the *CCN1* gene, on the other hand, we have observed an opposite effect. *CCN1* is crucial in controlling cell growth, specialization, programmed cell death, pulmonary blood vessel formation (28), and the development of fibrous tissue (29). Here, a 16% decrease was found in non–protein-coding isoform because of a retained intron in severe COPD and, therefore, a predicted increase in protein-coding transcripts. At the same time, DGE analysis has shown a downregulation of *CCN1* in severe COPD. It can be speculated that a decrease in mRNA production may be compensated by the production of fewer non–protein-coding AS transcripts. Therefore, the changes in gene expression of *CCN1* may not be translated to protein level or translated to a smaller degree than suggested by the results of DGE analysis.

The strength of our study design is that data were obtained from a large human cohort where participants were matched for age and smoking history, enabling us to examine with precision the association of COPD with AS. Our study also has some limitations. Particularly, our dataset contained more patients with severe COPD than patients with mild COPD and non-COPD control subjects. We attempted to address this issue by performing a permutation analysis. In addition, the possible variability in cell-type composition of bronchial brushings may affect transcriptomic findings, such as frequencies of AS events. Next, although nearly all our patients were ex-smokers, there was a

significant difference in pack-years between the groups. In addition, there was a sex imbalance among the groups. Part of the observed changes in AS may be linked to the differences in inhaled corticoid steroid (ICS) use between study groups, as suggested by analysis of AS in mild-to-moderate COPD between ICS and non-ICS groups (*see* Figure E6). However, such an intriguing possibility requires a separate investigation in a specifically designed experiment. It should be noted that because total RNA rather than poly(A)-selected libraries were used to generate the data, the increased intronic expression and the presence of immature RNAs are to be expected. Hence, the number of reads supporting retained introns may be inflated in our data. However, the increased intronic expression should equally affect all samples as samples were collected, processed, and analyzed as part of the same study, in a uniform manner. The other limitation of our analysis was that we relied on current protein annotation to evaluate whether the resulting AS transcripts are protein coding or not, which may result in over- or underestimation of the effect of AS on protein-coding capacity. Reliance on current gene annotation for functional interpretation of the AS effect limited us to only consider annotated splice junctions. In future studies, full-length transcriptome data would also allow one to consider novel splice junctions and their relation to the coding capacity of the corresponding isoform. In addition, when we evaluated the effect of differences in AS between patients with severe COPD and control subjects on the protein-coding capacity of affected genes, we did not take into consideration the magnitude of differences in relative proportions of AS transcripts, pulling together differential AS events with low differences ($<5\%$) and high differences ($>10\%$).

Changes in AS observed during COPD can be directly relevant to pathophysiological changes in the disease. However, it is still challenging to conclude whether the changes in AS dynamics between patients with mild and severe COPD observed in this study are a cause or consequence of COPD pathogenesis. To discriminate between these possibilities, future studies in COPD should focus not only on gene expression levels but also on differences in isoform compositions and subsequent functional changes on the protein level (e.g., caused by the inclusion or exclusion of isoform-specific protein domains or target sites for posttranslational modifications). Integrating gene expression and isoform regulation data will help us to get a more holistic view of molecular changes in COPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Agustí A, Vogelmeier C, Faner R. COPD 2020: changes and challenges. *Am J Physiol Lung Cell Mol Physiol* 2020;319:L879–L883.
2. Roessler FK, Benedikter BJ, Schmeck B, Bar N. Novel computational analysis of large transcriptome datasets identifies sets of genes distinguishing chronic obstructive pulmonary disease from healthy lung samples. *Sci Rep* 2021;11:10258.
3. de Vries M, Faiz A, Woldhuis RR, Postma DS, de Jong TV, Sin DD, *et al.* Lung tissue gene-expression signature for the ageing lung in COPD. *Thorax* 2017;73:609.
4. Brandsma C-A, Guryev V, Timens W, Ciconelle A, Postma DS, Bischoff R, *et al.* Integrated proteogenomic approach identifying a protein signature of COPD and a new splice variant of SORBS1. *Thorax* 2020;75: 180–183.
5. Faiz A, van den Berge M, Vermeulen CJ, Ten Hacken NHT, Guryev V, Pouwels SD. AGER expression and alternative splicing in bronchial biopsies of smokers and never smokers. *Respir Res* 2019;20:70.
6. Baralle FE, Giudice J. Alternative splicing as a regulator of development and tissue identity. *Nat Rev Mol Cell Biol* 2017;18:437–451.
7. Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet* 2008;40:1413–1415.
8. Bonnal SC, López-Oreja I, Valcárcel J. Roles and mechanisms of alternative splicing in cancer—implications for care. *Nat Rev Clin Oncol* 2020;17:457–474.
9. Biamonti G, Amato A, Belloni E, Di Matteo A, Infantino L, Pradella D, *et al.* Alternative splicing in Alzheimer's disease. *Aging Clin Exp Res* 2021;33: 747–758.
10. La Cognata V, D'Agata V, Cavalcanti F, Cavallaro S. Splicing: is there an alternative contribution to Parkinson's disease? *Neurogenetics* 2015; 16:245–263.
11. Newman JRB, Conesa A, Mika M, New FN, Onengut-Gumuscu S, Atkinson MA, *et al.* Disease-specific biases in alternative splicing and tissue-specific dysregulation revealed by multitissue profiling of lymphocyte gene expression in type 1 diabetes. *Genome Res* 2017;27: 1807–1815.
12. de Miguel FJ, Sharma RD, Pajares MJ, Montuenga LM, Rubio A, Pio R. Identification of alternative splicing events regulated by the oncogenic factor SRSF1 in lung cancer. *Cancer Res* 2014;74:1105–1115.
13. Saferali A, Yun JH, Parker MM, Sakornsakolpat P, Chase RP, Lamb A, *et al.*; COPDGene Investigators; International COPD Genetics Consortium Investigators. Analysis of genetically driven alternative splicing identifies FBXO38 as a novel COPD susceptibility gene. *PLoS Genet* 2019;15:e1008229.
14. Coomer AO, Black F, Greystoke A, Munkley J, Elliott DJ. Alternative splicing in lung cancer. *Biochim Biophys Acta Gene Regul Mech* 2019; 1862:194388.
15. Kong-Beltran M, Seshagiri S, Zha J, Zhu W, Bhawe K, Mendoza N, *et al.* Somatic mutations lead to an oncogenic deletion of met in lung cancer. *Cancer Res* 2006;66:283–289.
16. Deletang K, Taulan-Cadars M. Splicing mutations in the CFTR gene as therapeutic targets. *Gene Ther* 2022;29:399–406.
17. Thomas CG, Psarros C, Gekas A, Vandoros GP, Philippou A, Koutsilieris M. Alternative splicing of IGF1 gene as a potential factor in the pathogenesis of Peyronie's disease. *In Vivo* 2016;30:251–256.
18. Shen S, Park JW, Lu ZX, Lin L, Henry MD, Wu YN, *et al.* rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc Natl Acad Sci USA* 2014;111:E5593–E5601.
19. Barnes PJ. Senescence in COPD and its comorbidities. *Annu Rev Physiol* 2017;79:517–539.
20. Rutten EP, Gopal P, Wouters EF, Franssen FM, Hageman GJ, Vanfleteren LE, *et al.* Various mechanistic pathways representing the aging process are altered in COPD. *Chest* 2016;149:53–61.
21. Bhadra M, Howell P, Dutta S, Heintz C, Mair WB. Alternative splicing in aging and longevity. *Hum Genet* 2020;139:357–369.
22. Geuens T, Bouhy D, Timmerman V. The hnRNP family: insights into their role in health and disease. *Hum Genet* 2016;135:851–867.
23. West KO, Scott HM, Torres-Odio S, West AP, Patrick KL, Watson RO. The splicing factor hnRNP M is a critical regulator of innate immune gene expression in macrophages. *Cell Rep* 2019;29:1594–1609.e5.
24. Chen TM, Lai MC, Li YH, Chan YL, Wu CH, Wang YM, *et al.* hnRNPM induces translation switch under hypoxia to promote colon cancer development. *EBioMedicine* 2019;41:299–309.
25. Buro-Auriemma LJ, Salit J, Hackett NR, Walters MS, Strulovici-Barel Y, Staudt MR, *et al.* Cigarette smoking induces small airway epithelial epigenetic changes with corresponding modulation of gene expression. *Hum Mol Genet* 2013;22:4726–4738.
26. Alsafadi HN, Staab-Weijnitz CA, Lehmann M, Lindner M, Peschel B, Königshoff M, *et al.* An ex vivo model to induce early fibrosis-like

changes in human precision-cut lung slices. *Am J Physiol Lung Cell Mol Physiol* 2017;312:L896–L902.
27. Woldhuis R, De Vries M, Timens W, Van den Berge M, Oliver B, Heijink I, *et al.* Cellular senescence in lung fibroblasts from COPD patients is associated with altered extracellular matrix regulation [abstract]. *Am J Respir Crit Care Med* 2019;199:A3763.
28. Liu Y, Tang B-L, Lu M-L, Wang H-X. Astragaloside IV improves pulmonary arterial hypertension by increasing the expression of CCN1 and activating the ERK1/2 pathway. *J Cell Mol Med* 2023;27:622–633.
29. Zhu Y, Almuntashiri S, Han Y, Wang X, Somanath PR, Zhang D. The roles of CCN1/CYR61 in pulmonary diseases. *Int J Mol Sci* 2020;21: 7810.