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# Patients with multiple sclerosis who develop immunogenicity to interferon-beta have distinct transcriptomic and proteomic signatures prior to treatment which are associated with disease severity

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ABSTRACT

Anti-drug antibodies (ADA) reduce the efficacy of immunotherapies in multiple sclerosis (MS) and are associated with increased disease progression risk. Blood biomarkers predicting immunogenicity to biopharmaceuticals represent an unmet clinical need. Patients with relapsing remitting (RR)MS were recruited before (baseline), three, and 12 (M12) months after commencing interferon-beta treatment. Neutralising ADA-status was determined at M12, and patients were stratified at baseline according to their M12 ADA-status (ADA-positive/ADA-negative). Patients stratified as ADA-positive were characterised by an early dampened response to interferon-beta (prior to serum ADA detection) and distinct proinflammatory transcriptomic/proteomic peripheral blood signatures enriched for 'immune response activation' including phosphoinositide 3-kinase-γ and NFκB-signalling pathways both at baseline and throughout the treatment course, compared to ADA-negative patients. These immunogenicity-associated proinflammatory signatures significantly overlapped with signatures of MS disease severity. Thus, whole blood molecular profiling is a promising tool for prediction of ADA-development in RRMS and could provide insight into mechanisms of immunogenicity.

## 1. Introduction

Immunotherapies have revolutionised the treatment of autoimmune diseases, including multiple sclerosis (MS). A main caveat for immunotherapy effectiveness is the development of anti-drug antibodies (ADAs) which result from an undesirable immune response against the therapeutic (termed immunogenicity) [1–4]. Neutralising (n)ADA can block the biological activity of the drug, reduce treatment effectiveness, or exacerbate adverse reactions to treatment by altering drug pharmacodynamic properties [1,3,5].

*Abbreviations:* ADAs, Anti-drug antibodies; BMI, Body mass index; DEG, Differentially expressed gene; DMTs, Disease modifying therapies; EDSS, Expanded disability status score; FDR, False discovery rate; FC, Fold change; M, Month; MS, Multiple sclerosis; NPX, Normalised protein expression; padj, Adjusted p-value; RRMS, Relapsing remitting multiple sclerosis; SPMS, Secondary progressive multiple sclerosis..

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MS is a progressive, inflammatory, demyelinating, and neurodegenerative disease of the central nervous system [6,7]. Although there is no cure for MS, there are over 18 disease modifying therapies (DMTs) approved for relapsing remitting (RR)MS [8]. However, clinical trials investigating DMTs in progressive disease have yielded largely negative results, with only ocrelizumab indicated for primary progressive MS [6,9] and Siponimod for secondary progressive (SP)MS, which target the inflammatory component of the disease [10]. Instead, focus has relied on treatment during the RRMS phase since studies demonstrate that early and effective treatment with DMTs decreases the conversion rate from RRMS to SPMS and reduces the proportion of patients reaching irreversible damage [11,12]. Therefore, early treatment with an effective DMT in RRMS is of the utmost importance for the long-term prognosis of people with MS.

Immunogenicity is seen against many DMTs used to treat MS, including interferon-beta (IFNβ), alemtuzumab, natalizumab, and ocrelizumab [13]. IFNβ is still used widely globally as a first-line immunotherapy for many RRMS patients [14,15] and has a relatively high incidence of ADA (from 7 to 42% across IFNβ clinical trials) associated with a significant reduction of clinical effectiveness [16,17], making IFNβ an excellent candidate treatment to investigate signatures of immunogenicity. The incidence of nADAs to IFNβ varies across the different drug formulations: 28–47% for subcutaneous IFNβ-1b (Betaferon/Extavia), 13–25% for subcutaneous IFNβ-1a (Rebif) and 2–22% for intra-muscular IFNβ-1a (Avonex) [17]. However, irrespective IFNβ type, dose, or frequency of administration, once high nADA titres have been established, they persist for many years and render the therapy ineffective [16,17]. A recent observational study found that MS patients with high titre IFNβ nADAs had significantly increased annualised relapse rates, shorter time to first relapse and increased disease activity levels compared to nADA negative patients, likely due to the loss of IFNβ effectiveness [17]. This highlights the importance of early identification of nADA positive patients ('proactive monitoring'). However, nADA testing is not performed regularly when monitoring MS patients, rather patients are assessed for a loss in treatment efficacy ('reactive monitoring'). Since time spent on an ineffective treatment can place MS patients at risk of developing irreversible neurological damage and worse long-term outcomes this study aimed to identify early markers of immunogenicity in a prospective RRMS cohort and investigate how these markers may be associated with disease severity.

## 2. Materials and methods

### 2.1. Patients

Treatment naïve patients with RRMS ($n = 11$), diagnosed according to the revised McDonald criteria 2010 [18], were recruited from the General University Hospital in Prague from January 30, 2014 to January 22, 2016 as part of an observational clinical trial (Anti-Biopharmaceutical Immunization: Prediction and analysis of clinical relevance to minimize the risk of immunization in multiple sclerosis patients on interferon-beta treatment) which is registered in the EU Clinical Trials Register: https://www.clinicaltrialsregister.eu/ctr-search/trial/2012-005450-30/SE) (ABIRISK, www.abirisk.eu/). Patients ($n = 11$) were subsequently treated with either IFNβ-1a (Rebif) or IFNβ-1b (Betaferon/Extavia) as part of their normal standard of care (Table 1). Serum and whole blood RNA (Paxgene) was collected at three timepoints: Baseline, prior to starting IFNβ therapy (M0), three months (M3) and 12 months (M12) after starting treatment. Serum was tested for nADAs using a cell-based luciferase reporter gene assay [19]. Patients were classified as ADA-positive (ADApos) if they had a nADAs titre ≥320 U/mL at any point within 12 months of starting treatment [19,20]. Of the 11 RRMS patients, six were ADApos and five remained ADA-negative (ADAneg). Additionally, SPMS patients ($n = 8$), diagnosed according to the revised McDonald criteria [18], were recruited as part of the RELOAD study at University College London Hospital NHS Trust. SPMS patients were not on any DMTs at time of blood sampling. Demographic and clinical information were recorded, including sex, age, ethnicity, body mass index (BMI), smoking status, type, and dose of IFNβ, and expanded disability status score (EDSS) (Table 1). Smoking status was categorized as 'never smoked', 'ex-smoker, or 'current smoker'. Ethnicity was determined from study database (ABIRISK) or self-reported (RELOAD).

Ethical approvals for this work were obtained from the Medical Ethics Committee of the General University Hospital in Prague (125/12 and Evropský grant 1. LF UK-CAGEKID) and from the University College London Hospitals National Health Service Trust research ethics committee (18/SC/0323, RELOAD-MS study). All participants provided informed written consent in accordance with the Declaration of Helsinki.

**Table 1**
**Patient demographics**: Baseline demographic and clinical characteristics were compared between relapsing remitting (RR)MS patients ($n = 11$) who did or did not develop neutralising anti-drug antibodies (ADA) (ADApos, $n = 6$ vs ADAneg $n = 5$) to IFNβ treatment within 12 months, and in 8 secondary progressive (SP)MS patients. At time of blood sampling no SPMS patients were on disease modifying therapies. Statistical comparisons were made using [1]chi-squared, [2]Mann-Whitney U. Age and disease duration are given in years. Abbreviations: BMI, body mass index; EDSS, expanded disability status score; IFNβ-1a, Rebif; IFNβ-1b, Betaferon/Extavia; IQR, interquartile range; M0, month 0, before first treatment with IFNβ.

| | | RRMS ADAneg (n = 5) | RRMS ADApos (n = 6) | SPMS (n = 8) | *P*-value ADApos vs ADAneg | *P-value SPMS (n = 8) vs RRMS (n−11)* |
|---|---|---|---|---|---|---|
| Sex *n (%)* | Female | 3 (60) | 5 (83.3) | 6 (75) | 0.3869[1] | 0.2801[1] |
| | Male | 2 (40) | 1 (16.7) | 2 (25) | | |
| Age | Median (IQR) | 34 (3) | 34 (6.5) | 57.5 (11) | 0.7114[2] | 0.0005[2] |
| Disease Duration | Median (IQR) | 0.4 (0.6) | 5.9 (5) | 18 (7.5) | 0.0549[2] | 0.0003[2] |
| Ethnicity *n (%)* | White | 5 (100) | 6 (100) | 7 (87.5) | n/a | n/a |
| | Mixed | 0 (0) | 0 (0) | 1 (12.5) | | |
| BMI | Median (IQR) | 24.3 (1.9) | 25.6 (5.9) | 28.1 (7.5) | 0.6455[2] | 0.4715[2] |
| Smoking *n (%)* | Non-smoker | 2 (40) | 4 (66.7) | 3 (37.5) | 0.1967[1] | 0.0832[1] |
| | Quit smoking | 3 (60) | 1 (16.7) | 4 (50) | | |
| | Current smoker | 0 (0) | 1 (16.7) | 0 (0) | | |
| | Not disclosed | 0 (0) | 0 (0) | 1 (12.5) | | |
| DMT *n (%)* | IFNβ-1a | 3 (60) | 2 (33.3) | 0 (0) | 0.3765[1] | n/a |
| | IFNβ-1b | 2 (40) | 4 (66.7) | 0 (0) | | |
| | None | 0 (0) | 0 (0) | 8 (100) | | |
| EDSS at M0 | Median (IQR) | 2.0 (0.5) | 2.0 (0.4) | 6 (0) | 0.5222[2] | 0.0022[2] |
| Change in EDSS | Median (IQR) | 0 (0.0) | 0 (0.75) | n/a | 0.8572[2] | n/a |

### 2.2. RNA preparation sequencing and analysis

Whole blood RNA was isolated from patients with RRMS ($n = 11$) and SPMS ($n = 8$) using Qiagen PAXgene Blood RNA extraction kit. See **Supplementary Methods** for detailed description of RNA-sequencing methods. Differential gene expression comparisons performed were: Time-course analysis including RRMS patients ($n = 11$) at three different timepoints, to investigate response to IFNβ: 1) ADAneg-M0 vs ADAneg-M3; 2) ADAneg-M0 vs ADAneg-M12; 3) ADApos-M0 vs ADApos-M3; 4) ADApos-M0 vs ADApos-M12; 5) ADApos-M3 vs ADAneg-M3; 6) ADApos-M12 vs ADAneg-M12; Baseline analysis investigating differences in transcriptomic and proteomic profiles at baseline (M0) between RRMS patients (n = 11) and SPMS patients ($n = 8$): 1) ADApos-M0 vs ADAneg-M0; 2) SPMS vs ADApos-M0; 3) SPMS vs ADAneg-M0. Differentially expressed gene (DEG) lists are supplied in **Supplementary Data File 1**.

### 2.3. Proteomics

Serum samples from all patients were analysed using the Olink targeted 'immune response' panel (https://www.olink.com/content/uploads/2021/09/1051-v1.2-immune-response-panel-content-final.pdf) comprising 96 proteins. Data was standardised to normalised protein expression (NPX) values for relative quantification. NPX values were transformed into linear measurements, for more informative investigation of protein values. Differentially expressed protein lists are supplied in **Supplementary Data File 2**.

### 2.4. Data Analysis

The data analysis pipeline is summarized in **Fig. S1.** See **Supplementary Methods** for details of analysis methods used.

### 2.5. Statistical analysis

Genes which passed a threshold of both FDR adjusted $p$-value <0.05 (padj) and fold change (FC) ± 1.5 (log2FC: 0.585) were included in differential gene expression analysis**.** Proteomic values were assessed for FC, with $p$-values obtained from standard $t$-tests, which were not adjusted for multiple testing. $P$ values $P < 0.05$ were considered significant. Box plots for individual genes/proteins were plotted and mean +/-SD shown. All statistical analysis was performed using R programming.

## 3. Results

### 3.1. Transcriptomic and proteomic profiles detect early loss of response to IFNβ in ADA-pos RRMS patients

While changes in blood transcriptomic profiles after initiation of IFNβ treatment are described in patients with RRMS [16,21–23], they have not been investigated in the context of patients who go on to develop nADA. Therefore, longitudinal whole blood transcriptomic profiling was performed on matched samples from patients with RRMS at baseline (M0, before first treatment with IFNβ), and 3 months (M3) and 12 months (M12) after IFNβ treatment initiation. Patients were stratified by their nADA status determined at M12: ADApos ($n = 6$) and ADAneg ($n = 5$) (Table 1 for patient characteristics, Figs. 1 and **S1** for study analysis plan). There were no significant differences in baseline demographic or disease activity measures between patients in ADApos and ADAneg groups. Also, most patients in the ADApos group remained below the threshold for ADA-positivity at M3 (**Table S1**).

Differential gene expression was assessed between pre-and post-treatment samples stratified for ADA status as shown in Fig. 1. As expected, patients who remained ADAneg had a robust response to IFNβ at M3 (M3 vs M0) characterised by 713 upregulated and 250 downregulated genes (Fig. 2A), However, patients stratified as ADApos, had a muted response to IFNβ with far fewer differentially expressed genes (DEGs), 144 upregulated and 7 downregulated (Fig. 2B). Thus, while all patients demonstrated changes in gene expression in response to IFNβ treatment at M3, those patients stratified as ADApos had a lesser response, even before significant detectable ADA were present in most patients (**Table S1**). At M12, 486 upregulated and 189 downregulated genes were detected in ADAneg patients (M12 vs M0, Fig. 2C), but no DEGs were identified in ADApos patients at this timepoint (Fig. 2D), likely due to ADAs neutralising any effect of IFNβ. Interestingly, when DEGs from all comparisons were assessed across ADAneg and ADApos patients, 136 shared DEGs were identified (Fig. 2E). Pathway enrichment analysis of the 136 shared DEGs point to a signature of IFNβ response ("Interferon Alpha Response" and "Interferon Signalling") as well as pathways known to be upregulated in response to IFNβ in RRMS patients [16,22] including apoptosis-related pathways ("p53 transcriptional gene network" and "Apoptosis") (Fig. 2F). Overall, these results highlight that all patients had a molecular response to IFNβ therapy at early timepoints (M3) however this response was much stronger, more significant and sustained over 12 months in the ADAneg patients but abrogated by M12 in the ADApos patients.

The early differential response to IFNβ in ADApos versus ADAneg patients was further confirmed in proteomic analysis where 96 proteins from an immune response panel were analysed at M0 (baseline), M3, and M12 using the same four comparisons (Fig. 2G-J). As seen in the transcriptomic analysis, ADAneg patients had a stronger or more sustained molecular reaction to IFNβ compared to ADApos patients, and ADApos patients lost signatures of response to treatment by M12. This is particularly evidenced by two IFN-related proteins, DDX58 (DEAD box protein 58) and LAMP3 (Lysosomal Associated Membrane Protein 3), which were identified in both the proteomic and gene expression

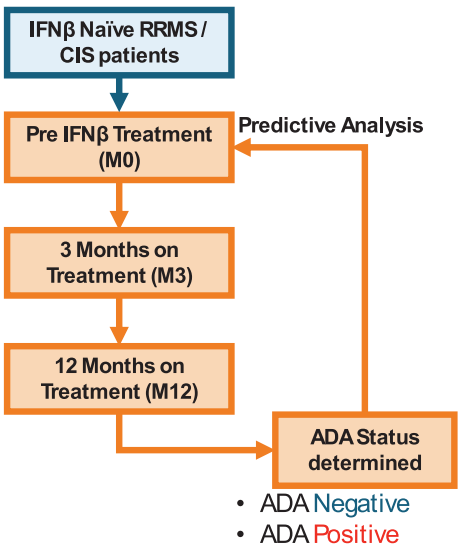


**Fig. 1. Stratification of treatment naïve patients diagnosed with RRMS or CIS.** Patients diagnosed with relapsing remitting multiple sclerosis (RRMS) or clinically isolated syndrome (CIS) according to revised McDonald criteria 2010 and treatment naïve for IFNβ were recruited and treated as standard of care by their treating clinician. Patient and clinical data and biological samples were collected at baseline (month, M0), M3 and M12 following treatment initiation. Serum was tested for neutralising and binding anti-drug antibodies (ADA). Patients were stratified according to their ADA status at M12. ADA positivity was defined according to the ABIRISK guideline [19,24]: *ADA-positive = positive for binding ADA and/or had a neutralising ADA titre ≥ 320 U/mL within 12 months of starting treatment.* Most patients first developed ADA after 6 months (M6) on IFNβ treatment (See Table S1). ADA-positive / ADA-negative stratification at M12 was applied to patients at baseline (M0, prior to first treatment) and all analysis was performed based on this stratification.

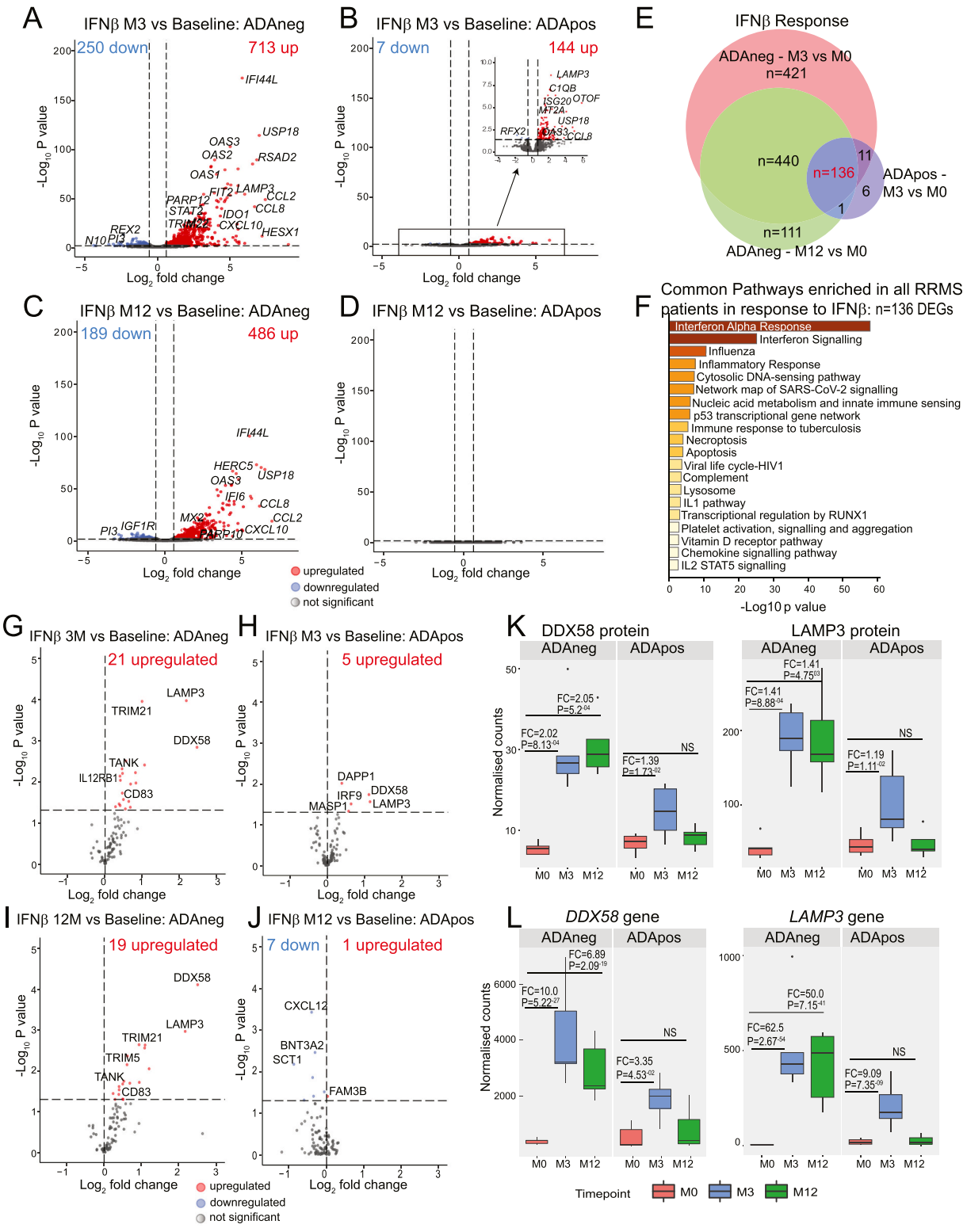


**Fig. 2. Patients who go on to develop ADA at M12 have a muted response to IFNβ compared to patients who remain ADAneg.** RRMS patients were stratified according to the detection of neutralising anti-drug antibodies at M12 following initiation of IFNβ therapy (ADApos/ADAneg). **(A-D)** Volcano plots showing differentially expressed genes (DEGs) where each gene is plotted according to its -log10 adjusted *p*-value (padj), y-axis, and log2fold change, x-axis. Genes were considered up/down-regulated at fold change +/− > 1.5, and significant at padj <0.05. **(A)** ADAneg: M3 vs M0; **(B)** ADAneg: M12 vs M0; **(C)** ADApos: M3 vs M0; **(D)** ADApos: M12 vs M0. **(E)** Venn analysis comparing overlapping DEGs for comparisons in A-C. **(F)** Pathway enrichment analysis using for overlapping DEGs ($n = 136$) from E. **(G-J)** Proteomic response to IFNβ treatment in RRMS patients comparing protein concentrations in ADApos and ADAneg patients. Volcano plots summarising differences in protein concentration for **(G)** ADAneg: M3 vs M0 **(H)** ADAneg: M12 vs M0 **(I)** ADApos: M3 vs M0 **(J)** ADApos: M12 vs M0. **(K-L)** DDX58 and LAMP3 protein concentration **(K)** and gene expression **(L)** demonstrated a difference in response to treatment between ADApos and ADAneg patients. Refer to Supplementary Data File 1.

analysis (Fig. 2K-L). The early muted effect of INFβ in ADApos patients was further illustrated when 17 genes and proteins previously associated with response to IFNβ were investigated within the transcriptomic dataset, including eight IFN-responsive genes [21,23], eight apoptosis genes [16,22], and LAMP3 which correlates directly with IFNβ activation [25] (**Table S2, Fig. S2A**). While most of these genes were significantly upregulated at M3 compared to baseline (padj <0.05), regardless of ADA status, the gene expression was significantly higher in ADAneg compared to ADApos patients at M3 for most genes. This difference was large and significant enough that a gene score could be

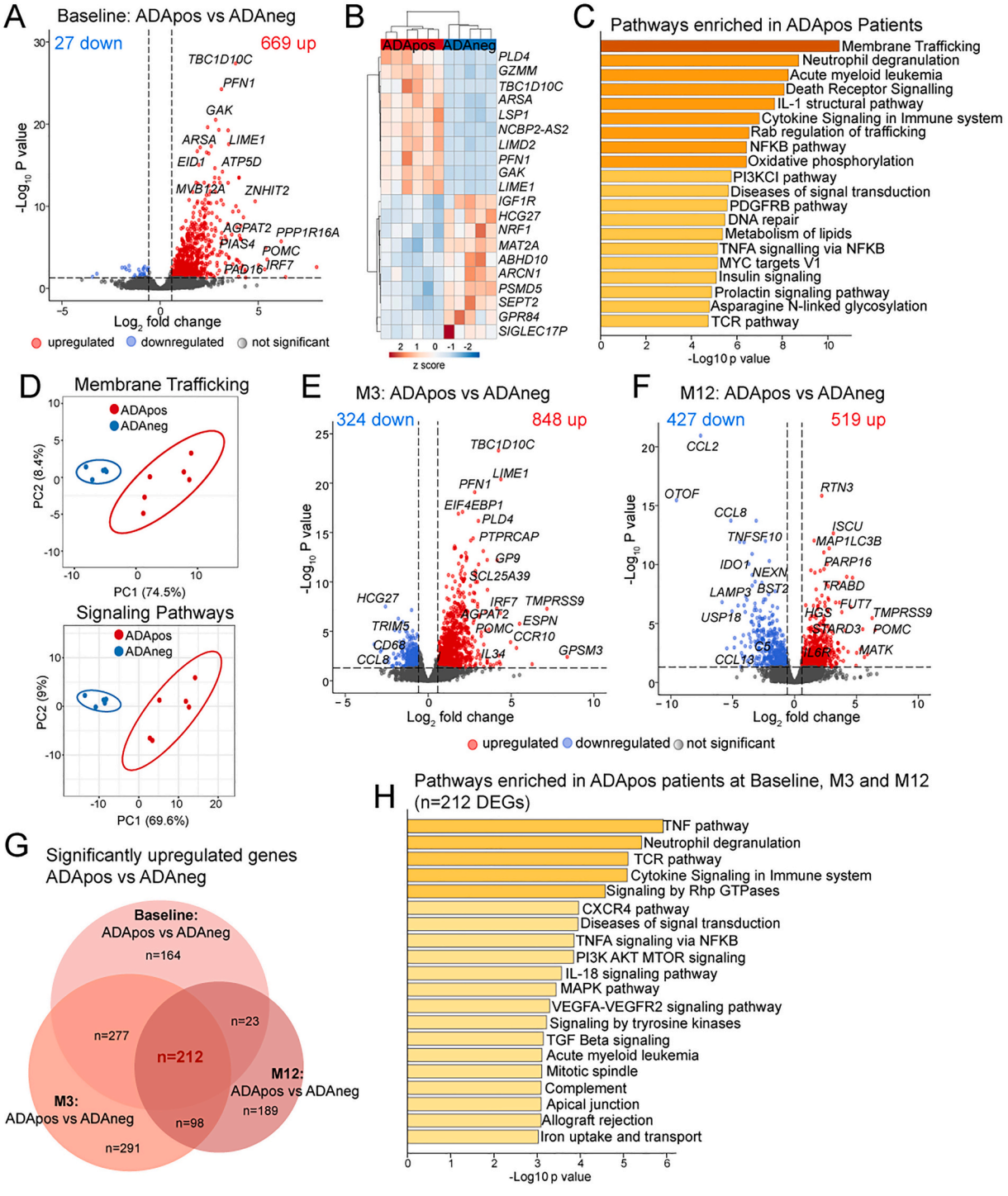


**Fig. 3. Transcriptomic profile differentiates ADApos from ADAneg patients prior to starting IFNβ therapy: (A)** Volcano plot showing differentially expressed genes (DEGs) between RRMS patients stratified according to their neutralising anti-drug antibody status at M12 after treatment initiation with IFNβ (ADApos / ADAneg). DEGs are plotted according to -log10 adjusted *p*-value (padj), y-axis, and log2fold change, x-axis. Genes were considered up/down-regulated at fold change+/− > 1.5, and significant with padj threshold of <0.05. **(B)** Heatmap of top 10 significantly up and down regulated genes (see Table S2). **(C)** Pathway enrichment analysis showing top 20 pathways based on upregulated DEGs (see Fig. S3). **(D)** Principal component analysis (PCA) of genes within the membrane trafficking or signalling pathways from (C). **(*E*-F)** Volcano plots showing DEGs comparing ADApos patients to ADAneg patients at M3 **(E)** and M12 **(F). (G)** Venn analysis of upregulated genes identifying DEGs upregulated at all timepoints. **(H)** Pathway enrichment analysis of commonly upregulated genes ($n = 212$) from (G).

constructed to classify patients into ADA status using the fold changes in the IFN-response genes from M0 to M3 (**Table S2 and Fig. S2B**). Together, these results show that the full effect of IFNβ treatment is inhibited in RRMS patients that go on to develop ADA from as little as M3 following treatment initiation, even though there are no significant detectable ADA in the serum at that time (**Table S1**).

### *3.2. Patients who develop ADA by M12 have distinct transcriptomic and proteomic profiles prior to treatment initiation*

Since patients stratified according to their M12 ADA status had striking differences in their early molecular responses to IFNβ treatment, the baseline (M0) transcriptomic and proteomic profiles of these patients were further investigated to find potential predictive signatures of immunogenicity. A comparison between RRMS patients stratified as ADApos and ADAneg prior to first IFNβ administration (M0) found 669 upregulated and only 27 downregulated DEGs (Fig. 3A). The top 10 most significantly up- and down-regulated genes clearly stratified ADApos from ADAneg patients (Fig. 3**B, Table S3**). Pathway enrichment analysis of the upregulated DEGs identified pathways associated with immune activation including: 'membrane trafficking' ($p = 3.12E-11$, enrichment score 3.5) and 'Rab (Ras-associated binding proteins) regulation of trafficking', ($p = 2.92E-07$, enrichment score 5.9) (Fig. 3C). Eleven of the top 20 regulated pathways were associated with cell signalling, notably 'NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells) Pathway' ($p = 3.75E-07$; enrichment score 16), 'PI3KCl (phosphoinositide 3-kinase Class I) Pathway' ($p = 1.76E-06$; enrichment score 9.1), and 'TNFA (tumour necrosis factor-α) Signalling via NFκB' ($p = 7.03E-06$; enrichment score, 4.1). Class I PI3Ks are known to play an important role in immune cell signalling including B-cell and T-cell receptor signalling, T-cell survival, proliferation, and differentiation [26], pathways that were significantly upregulated in ADApos patients (**Fig. S3**). DEGs associated with both membrane trafficking ($n = 42$) and cell signalling pathways ($n = 156$) were also able to stratify patients by ADA status (Fig. 3D) and support the importance of immune cell migration across the blood brain barrier and cytokine signalling in the pathology of MS [16,27].

The transcriptomic differences between ADApos and ADAneg patients were maintained after treatment at M3 and M12 (Fig. 3E-F). Importantly, a subset of upregulated DEGs consistently discriminated between ADApos and ADAneg at all timepoints ($n = 212$ genes) (Fig. 3G). These genes were enriched within various signalling pathways including the 'TNF pathway', 'TNFα signalling via NFκB', and 'PI3K-AKT mTOR (mammalian target of rapamycin) signalling', matching the signature discriminating between ADApos from ADAneg patients at baseline (M0) (Fig. 3H). This suggests that patients who go on to develop ADA have a pro-inflammatory signature prior to starting treatment (Fig. 3C), that is maintained throughout the treatment time course.

### *3.3. Signatures of immunogenicity at baseline (M0) overlap with signatures of disease severity*

Some of the signatures of immunogenicity found both at baseline and following treatment with IFNβ also overlapped with published biomarkers of SPMS. For example, PI3Kγ and NFκB signalling pathways, were upregulated in ADApos patients at all timepoints (Fig. 3H) and have also been associated with MS incidence and disease severity [28–31]. On an individual gene level, MMP9 (Matrix Metalloproteinase 9), a well-established marker of MS disease progression, was upregulated in ADApos patients at all timepoints (**Supplemental Data File 1**). Interestingly, increased expression of RAB32 in active lesions of MS patients has been described previously [32] and RAB genes and the pathway 'RAB regulation of membrane trafficking' were also upregulated in ADApos patients (Fig. 3C). Therefore, the potential overlap between patients with RRMS stratified as ADApos and patients who have progressed to SPMS was investigated further.

Patients with RRMS stratified by nADA status at baseline (M0 prior to starting IFNβ therapy), were compared to eight SPMS patients using whole blood transcriptomics and proteomics. Differential gene expression analysis between SPMS vs ADAneg RRMS patients identified multiple differences (776 genes enriched in SPMS patients, and 91 genes enriched in ADAneg RRMS) (Fig. 4A), while in SPMS vs ADApos RRMS patients only nine genes were enriched in SPMS, and 21 genes enriched in ADApos RRMS patients (Fig. 4B). This demonstrated a potential similarity between ADApos RRMS patients and SPMS patients, which was confirmed by comparing the overlap between genes enriched in the SPMS vs ADAneg comparison and those enriched in the ADApos vs ADAneg RRMS comparison ($n = 515$ DEGs, Fig. 4C). In addition, patients could be grouped as either SPMS, ADApos RRMS, or ADAneg RRMS (Fig. 4D-E) using only the list of 196 membrane trafficking and immune signalling genes upregulated in ADApos RRMS patients compared to ADAneg patients (Fig. 3D). Gene counts of MS biomarkers including MMP9 and RAB35 [16,32] followed a similar trend, wherein the gene expression between SPMS and ADApos RRMS patients were comparable, but significantly different from ADAneg RRMS patients (Fig. 4F). These results were confirmed using proteomics analysis (**Fig. S4A—B**).

Finally, the PI3Kγ and NFκB pathways, as summarized in Fig. 5A were of particular interest. These pathways are not only linked to MS and disease progression, but also feature among the top 20 pathways to be upregulated in RRMS patients stratified as ADApos compared to ADAneg. The genes involved in these pathways were further investigated in SPMS, ADApos, and ADAneg RRMS patient groups (Fig. 5B-C) and were again confirmed by proteomics (Fig. 5D**, Fig. S4C**). Thus, these results demonstrate the transcriptomic and proteomic overlap between immunogenicity and disease progression in patients with MS.

## 4. Discussion

Although DMTs such as alemtuzumab, natalizumab, and ocrelizumab are more effective at modifying disease progression compared to IFNβ, the preferable safety profile and convenient administration of IFNβ mean that it remains commonly used globally as a first-line treatment for patients with RRMS [9,33,34]. While previous research has identified various molecular markers of response to IFNβ treatment in RRMS patients [16,21–23], these markers have not been investigated in the context of nADAs. In this study we examined longitudinal transcriptomic and proteomic signatures of response to IFNβ therapy in treatment naïve RRMS patients at M0, M3, and M12, stratified by M12 nADA status. This approach both validated IFNβ-response markers in ADAneg patients and characterised how these response markers changed in ADApos patients. ADApos patients had a much weaker response to IFNβ during the early phases of treatment before significant detectable nADA titres were observed, but also had distinct transcriptomic and proteomic signatures prior to first treatment with IFNβ, associated with elevated immune activation. Finally, signatures associated with nADA development significantly overlapped with signatures of disease severity identified in patients with SPMS.

All markers known to be upregulated in response to IFNβ therapy [16,21–23] were also upregulated in RRMS patients at M3, regardless of nADA status. However, the extent of these changes was significantly dampened in ADApos patients compared to ADAneg at M3 and were completely abrogated by M12. This effect was mirrored in the proteomic analysis. These results are of particular interest because between M0 and M3, most ADApos patients did not pass the criteria for nADA positivity. Therefore, the early difference in response to IFNβ in ADApos patients might not be due to the neutralising effects of ADA, but instead demonstrate an altered immune response at baseline that is predictive of future nADA development.

A main aspect of this work was to identify molecular signatures predictive of immunogenicity to biopharmaceuticals prior to treatment commencement. We show that prior to IFNβ therapy, ADApos patients

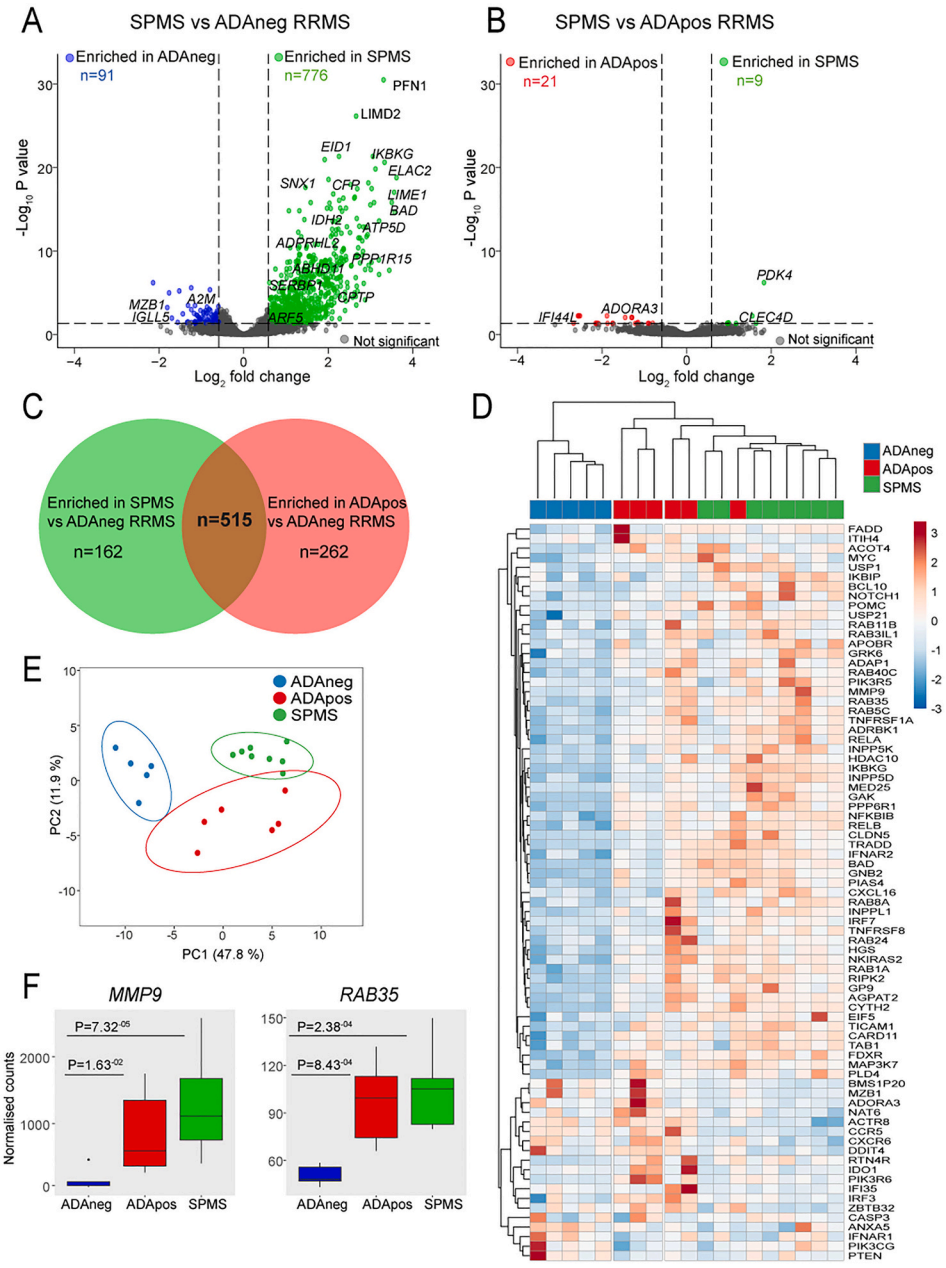


**Fig. 4. Transcriptomic signature of immunogenicity overlaps with signature of disease severity in MS.** Differentially expressed gene (DEG) analysis comparing SPMS with RRMS patients at baseline (M0, prior to IFNβ treatment) stratified according to their neutralising anti-drug antibody status at M12 after treatment initiation with IFNβ (ADApos / ADAneg) **(A)** SPMS vs ADAneg RRMS at M0 **(B)** SPMS vs ADApos RRMS at M0. **(C)** Venn diagram comparing DEGs that pass padj threshold identified in SPMS vs M0 ADAneg RRMS and M0 ADApos vs ADAneg RRMS patients. **(D-E)** DEGs enriched in both SPMS vs ADAneg RRMS and M0 ADApos vs ADAneg RRMS patients (n = 515 from C) were used to cluster patients using unsupervised hierarchical clustering **(D)** and principal component analysis **(E). (F)** Key biomarkers MMP9 (matrix metallopeptidase 9) and RAB35 (member RAS oncogene family) were investigated in further detail, where gene counts confirm clustering results, padjusted *p* values.

had a pro-inflammatory gene/protein signature. For example, upregulation of RAB proteins, is known to regulate the transport of immune receptors and secretion of chemokines and cytokines [35]. Interestingly, RAB proteins can predict immunotherapy response in patients with colorectal cancer [36]. Other work performed as part of the ABIRISK consortium [37] support that patients can be stratified prior to first treatment with immunotherapy. A genome-wide association study (GWAS) investigating clinicogenomic factors of immunogenicity in rheumatoid arthritis (RA), MS, and inflammatory bowel disease suggested a relationship between C-X-C motif chemokine ligand 12 production and ADA development, independent of disease phenotype, and provided evidence for the association of HLA-DQ-05 in ADA development [24]. The results also identified that tobacco smoking and infection during treatment were associated with increased risk of ADA development, whereas treatment with immunosuppressants and antibiotics were associated with decreased risk of ADA development [24]. Immune factors predictive of ADA development have also been described including a significant reduction of the frequency of signal regulatory protein (SIRP)a/b expressing memory B-cells in adalimumab-treated RA patients who went on to develop ADA [38] and activated NOTCH2 signalling pathway and increased frequency of proinflammatory monocyte subsets in RRMS patients prior to starting treatment with IFNβ who then go on to develop nADA [39]. In addition, serum metabolomic signatures can predict future ADA development, as

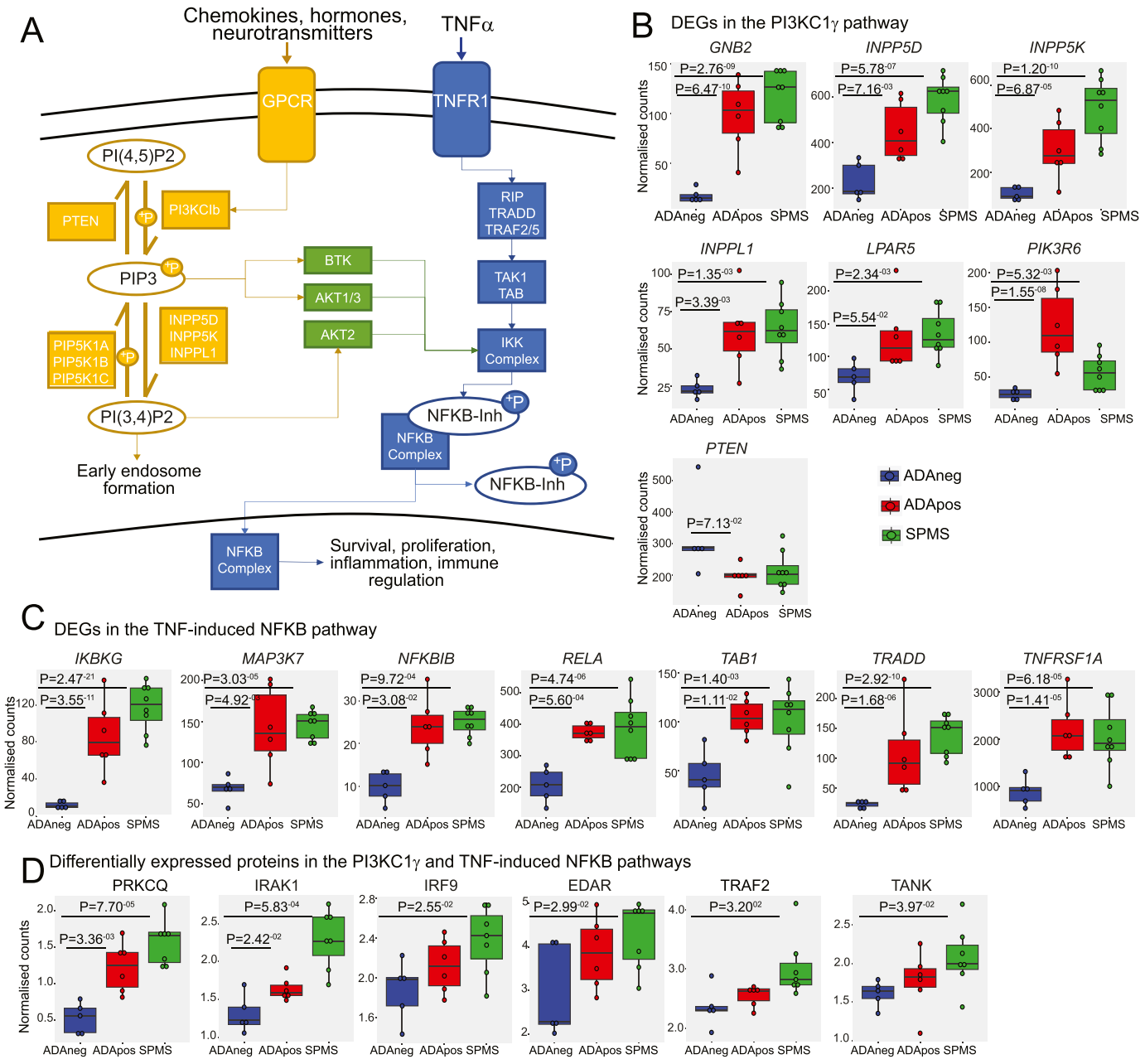


**Fig. 5. Signalling pathway signature in RRMS and SPMS. (A)** Schematic summary of the overlap of PI3KCIγ (phosphoinositide-3 kinase-class I-γ) and TNFα (tumour necrosis factor-α) induced NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathways. **(B—C)** Box plots visualising DEGs involved in the PI3KCIγ (B) and the TNF induced NFκB signalling pathways identified in this study (C) Adjusted *p*-values for the ADAneg vs ADApos and ADAneg vs SPMS comparisons shown. See Supplementary data file 1. **(D)** Transcriptomics results were validated using proteomics, with several proteins within the PI3KCIγ and TN- induced NFκB signalling pathways identified including: PRKCQ, Protein Kinase C Theta; IRAK1, Interleukin 1 Receptor Associated Kinase 1; IRF9, interferon regulatory factor 9; EDAR, Ectodysplasin A Receptor (mediates the of NFκB activation); TRAF2, TNF Receptor Associated Factor 2; TANK, TRAF Family Member Associated NFKB Activator. Significant *P* values shown. See Supplementary data file 2.

well as identify early changes in response to IFNβ in ADApos patients as described here [40]. Collectively these studies provide evidence that ADA can be predicted prior to starting treatment.

Anti-Type-I interferon antibodies are described in other conditions including lupus, severe COVID-19 infection and autoimmune polyendocrinopathy syndrome type-I (APS-1) [41]. In APS-1, anti-cytokine antibodies are generated as a result of defects in T cell immune regulation [41], this is in keeping with known mechanisms of anti-drug development where drug-responsive T helper cells become activated in the context of reduced regulatory T cell function [42]. In this study, patients stratified as ADApos were characterised by an activated immune profile, which existed prior to first drug administration. While a defect in immune regulatory pathways was not detected in these patients, such a defect predisposing patients to ADA development cannot be ruled out and deserves further investigation.

Our results also support previous findings that ADAs against IFNβ are associated with disease progression/severity [4,17]. It is known that patients with RRMS who develop ADAs, and patients with SPMS both fail to respond to IFNβ treatment [6,9,43] suggesting a potential pathogenic/mechanistic overlap. Indeed, at baseline there was a large overlap in significantly enriched genes and proteins between SPMS and ADApos RRMS patients. Furthermore, various MS disease severity markers were shared between SPMS and ADApos RRMS patients. This includes, RAS related protein RAB32 which is associated with MS

pathology, where increased expression of RAB32 is seen in active lesions in MS patients [32], suggesting a role for RABs in an inflamed central nervous system. In addition, genetic association studies identified variants and altered expression levels of clathrin-mediated endocytosis (a process regulated by RABs) in Alzheimer's disease, while altered expression of endocytic components are associated with Parkinson's disease [44]. PI3Kγ is also linked with various autoimmune disorders, including MS, systemic lupus erythematosus and RA [45]. Inhibition of PI3Kγ in mouse models of MS lead to an improvement of clinical scores [28,30] and reduction in disease severity [46], thus demonstrating PI3Kγ as a marker of MS pathogenesis. Meanwhile, NFκB is activated in many cell types in MS, including T-cells, macrophages, astrocytes, oligodendrocytes, and neurons [29,31]. In experimental models of MS, mice deficient in NFκB1 had reduced disease severity and CNS inflammation [31]. Additionally, GWAS have identified MS susceptibility loci in various key members of NFκB signalling, including TNFR1 and Mucosa-Associated Lymphoid Tissue Lymphoma Translocation (MALT) 1 [29]. This demonstrates that the pathways upregulated in ADApos patients in this study were not only signatures of immune activation, but also of MS disease pathology and severity. This is of particular interest since all RRMS patients included in this study at baseline were recently diagnosed and treatment naïve. The upregulated markers were unaffected by IFNβ treatment, as their gene expression remained upregulated at all timepoints, suggesting that the signatures of immunogenicity, which are sustained throughout treatment with IFNβ, may overlap with signatures of MS disease progression/severity. This theory is further supported by the upregulation of MMP9 at all timepoints, a well-established marker of MS disease progression [16]. While the association between disease severity and immunogenicity could be due to the lack of treatment efficacy due to nADA [17,47], the molecular similarities between ADA development and disease progression warrant further investigation.

This study included patients treated with IFNβ injected subcutaneously (Rebif/Beterferon). While subcutaneous drug administration is a convenient and cost effective delivery method [48,49], the complex immune environment of the skin, comprising both the innate and adaptive arms of the immune response [50,51], can predispose a patient towards immunogenicity in some cases [52]. Indeed, the incidence of nADAs to IFNβ is greater in patients receiving subcutaneous compared to intra-muscular IFNβ drug formulations [17,53]. The skin microenvironment is primed to respond to pathogens and injury [50,51], and in the same way biopharmaceutical proteins can be taken up and processed by professional antigen presenting cells either at the site of injection or draining lymph nodes. Anti-drug responses could be generated in susceptible individuals in the context of a more pro-inflammatory environment. The data presented in this manuscript supports this concept in that patients who develop ADAs had a more pro-inflammatory systemic immune profile which could reflect the situation in the skin, especially since the skin is populated by migratory as well as resident immune cells [50,51]. Therefore, it is important to understand the mechanisms of immunogenicity development using subcutaneous drug delivery compared to other methods including, intra-muscular and intravenous administration, since some MS therapeutics including ofatumumab and natalizumab are now available for subcutaneous delivery [48,49].

As well as route of administration, the impact of sex could influence the development of anti-drug responses. As with many autoimmune conditions, MS affects females more than males with a ratio of 2–3:1 [54]. Furthermore, females with MS have higher rates of inflammatory relapses, while males have higher risk of progressive disease and worse CNS damage. These sex-associated disease profiles are likely associated with the known differences in innate and adaptive immune responses between sexes where females have more robust immune responses compared to males [55]. Such sexual dimorphism can be beneficial when considering response to vaccines in females, but could also predispose to females to anti-drug responses [56]. Notably, sex was not an important factor in driving anti-drug responses within the ABIRISK cohorts, where patient and demographic factors were assessed in a prospective multicohort study comprising four autoimmune diseases (MS, RA, Crohn's disease, and ulcerative colitis) treated with eight different biopharmaceuticals [24]. Similarly, sex did not influence prediction of immunogenicity to IFNβ in patients with MS in a serum metabolomic analysis [40], and overall, despite some studies reporting increased in immunogenicity to IFNβ in females compared to males [53], there is currently no evidence of sex-based differences influencing response to DMTs [57].

The study had some limitations. We were unable to unravel any potential influence of sex on ADA-responses due to the small sample size. In addition, patients with SPMS and RRMS were recruited from different geographical regions, which could influence the results through potential differences in genetic background and environmental factors underlying the MS pathogenesis between these two groups. However, the patients with SPMS had previously clinically presented with RRMS and over time ceased to have relapses and developed progression independent of relapses. It therefore makes sense that the ADApos RRMS patients had a similar molecular profile to SPMS patients and supports similarities in immune activation which could be a predictor of both ADA and disease severity.

## 5. Conclusions

In conclusion, this study identified molecular signatures associated with future nADA development in patients with RRMS prior to first treatment with IFNβ and supports that biomarkers predicting future immunogenicity to DMTs could be developed for patients with RRMS. Such biomarkers could improve future disease management and clinical trial design. This study also highlights important signatures of immunogenicity which overlap with signatures of disease progression/severity. These findings together demonstrate how multi-omic markers could be used to aid treatment decisions in patients with RRMS [58].

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The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

## CRediT authorship contribution statement

**Leda Coelewij:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Marsilio Adriani:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Pierre Dönnes:** Writing – review & editing, Supervision, Resources, Methodology, Data curation. **Kirsty E. Waddington:** Writing – review & editing, Investigation, Formal analysis. **Coziana Ciurtin:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Eva Kubala Havrdova:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Rachel Farrell:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Petra Nytrova:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Inés Pineda-Torra:** Writing – review & editing, Supervision, Investigation, Funding acquisition,

Conceptualization. **Elizabeth C. Jury:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

*MA* is now employed by Nxera Pharma UK, Translational Sciences, Granta Park Steinmetz Building, Cambridge, United Kingdom. *KEW* is now employed by Nucleome Therapeutics, Oxford, United Kingdom. *EKH* has received honoraria/research support from Biogen, Merck Serono, Novartis, Roche, and Teva; has served as a member of advisory boards for Actelion, Biogen, Celgene, Merck Serono, Novartis, and Sanofi Genzyme; has been supported by the Czech Ministry of Education – project Cooperatio LF1, research area Neuroscience, and the project National Institute for Neurological Research (Programme EXCELES, ID project No LX22NPO5107) – funded by the European Union-Next Generation EU. *RF* has received speaker honoraria and hospitality from Merck, Biogen, TEVA, Novartis, Genzyme, Abbvie, Merz and Ipsen. *PN* has received speaker honoraria and consultant fees from Biogen, Novartis, Merck, and Roche. The remaining authors (*LC, PD, CC, IPT, ECJ*) declare no competing interests.

## Data availability

Normalised gene counts and protein expression data are available in Supplementary Data Files 1 and 2. Whole blood RNA-sequencing data will be deposited at Gene Expression Omnibus and proteomic data will be deposited at Mendeley. Both datasets will publicly available as of the date of publication.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2024.110339.

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