Research paper

Identification of two highly antigenic epitope markers predicting multiple sclerosis in optic neuritis patients

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ARTICLE INFO

Article History:
Received 3 June 2020
Revised 10 December 2020
Accepted 6 January 2021
Available online xxx

Keywords:
Multiple sclerosis
Optic neuritis
Herpesvirus
Antigen
CMV
EBV

ABSTRACT

Background: Optic neuritis (ON) can occur as an isolated episode or will develop to multiple sclerosis (MS) a chronic autoimmune disease. What predicts ON progression to MS remains poorly understood.

Methods: We characterised the antibody epitope repertoire in three independent clinical cohorts (discovery (n = 62), validation (n = 20) and external cohort (n = 421)) using mimotope variation analysis (MVA), a next generation phage display technology to identify epitopes that associate with prognosis of ON.

Findings: We observed distinct epitope profiles for ON, MS and the controls, whereas epitope repertoires of sera and CSF were highly similar. Two unique and highly immunogenic epitopes A and B were detected in subjects with ON progressing to MS. These epitopes A and B were strongly associated with herpesviral antigens (VCA p18 of Epstein-Barr virus (EBV); gB of Cytomegalovirus (CMV)), ROC addressed 75% of MS subjects with ON onset correctly (at 75% sensitivity and 74.22% specificity) based on the two-epitope biomarker analysis.

Interpretation: This is the first report on epitope diagnostics for MS employing the unbiased strategy of MVA for identification of novel immunological features of disease.

Funding: The Estonian Ministry of Education, The Estonian Research Council (PRG573, PRG805 and PSG691), H2020-MSCA-RISE-2016 (SZTEST), H2020-NMBP-2017 (PANBIORA), Helsinki University Hospital, Mary and Georg C. Ehrnrooth, Finnish Eye, Sigrid Jusélius and Magnus Ehrnrooth Foundations.

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1. Introduction

Multiple sclerosis (MS) is the most prevalent chronic inflammatory disease of the central nervous system (CNS). Most patients present with relapsing MS, with episodes of relapse and remission phases, whereas ~10% have a progressive course from the onset.

The causes of MS are not known. Associations with HLA-DRB1*15:01 (HLA class II) and HLA-A*02:01 (class I) along with more than 200 genetic variants have been reported to influence the risk of MS [1]. Female gender, Scandinavian/Celtic descent, low childhood vitamin D status, adolescent obesity, smoking, and infectious background — all include major risk factors of MS (reviewed in [2]). MS is considered an autoimmune disease, but the antigens of CNS targeted by T and B cells are currently largely unknown (reviewed in [3]). About 90% of patients with MS have lgG oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) [4]. OCBs are also common in other types of CNS inflammation [4,5]. Reactivity of OCBs and intrathecal antibodies against bacterial [e.g., Chlamydia pneumoniae [6-8]], human herpesvirus (Epstein-Barr virus (EBV), Human Herpesvirus 6 (HHV-6)) and other viral (measles, rubella, and zoster (“MRZ”) [9-14]) antigens have been reported in patients with MS, although underlying mechanisms remain unclear.

The optic nerve is one of the major targets in MS. About 20% of MS patients present with optic neuritis (ON) as one of the first symptoms, whereas during the course of the disease ON may occur in 50% of patients. In a follow-up of a large cohort of ON patients the risk of developing MS after ON was 30% at 5 years, close to 40% at 10 years, and 50% after 15 years [15].
2. Methods

2.1. Study population

The discovery cohort included CSF and sera/plasma samples of 24 treatment-naïve Finnish patients who initially received the diagnosis of ON and 38 controls (Table 1). Fifteen of the ON patients were subsequently diagnosed with relapsing MS (ON\textsuperscript{MS}) during the median follow-up time of 52 months (range 38–69 months), whereas 9 patients did not develop MS (denoted ON\textsuperscript{ON}). Using standard isoelectric focusing and agarose gel electrophoresis OCBs were found in all CSF samples of the discovery cohort. In the ON\textsuperscript{ON} group the average number of CSF-specific OCBs was 12; in the ON\textsuperscript{MS} group the average number of CSF-specific OCBs was 15 (the numbers of CSF-specific OCBs observed are in Figure S1a).

The validation cohort included, sera/plasma and CSF samples collected at diagnostic phase (treatment-naïve) of 20 Finnish patients with relapsing MS, out of which 10 presented with ON (MS\textsuperscript{ON}) and 10 with other symptoms (MS\textsuperscript{other}) (Table 1). All patients with MS in the discovery and validation cohorts fulfilled McDonald 2005 and 2017 criteria, their clinical features on baseline EDSS, visual functional score and brain MRI findings are summarised in Table S1 and OCBs in the CSF on Figure S1a.

The diagnostic model using ROC analysis was tested in an independent external cohort of Estonians including healthy individuals (n = 229, Table 2) and in subjects with different ICD-10 diagnosis codes, but without any notification of demyelinating disease (n = 192, Table 2).

2.2. Ethics statement

Patients for this study were recruited at the Department of Neurology and Department of Ophthalmology of Helsinki University Hospital, Finland. Written informed consent was obtained from all study participants. This study was pre-approved by the regional ethics committee at the Helsinki University Hospital (Dno 83/13/03/01/2013), Ethics Review Committee on Human Research of the University of Tartu, Estonia: 212T-24 (issued 13.03.12), 177/T-2 (issued 15.12.08), 211/M-22 (issued 23.01.12) and 281/T-5 (issued 16.04.18) and from Tallinn Medical Research Ethics Committee 1161 (issued 13.09.2007). The healthy control (HC) samples were from donors of the North Estonia Medical Blood Centre (Tallinn, Estonia).

2.3. Mimotope variation analysis (MVA)

For qualitative and quantitative characterisation of antibody epitopes from blood sera/plasma and CSF samples, we used the MVA method as described previously [16]. Analysis included discovery (sera/plasma and CSF samples; n = 62), validation (sera/plasma and CSF; n = 20) and independent external (sera/plasma; n = 421) cohorts. On average, MVA generated 3 million peptide sequences per sample.

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Table 1

Description of clinical samples studied by MVA.

| Characteristics         | Discovery cohort | Controls | Validation cohort |
|-------------------------|------------------|----------|-------------------|
|                         | ON\textsuperscript{ON} | ON\textsuperscript{MS} | CTRL | CTRL | MS\textsuperscript{ON} | MS\textsuperscript{other} |
| Group size (n)          | 9*                | 15**                | 27   | 11   | 10                          | 10                          |
| Diagnose on sample collec| ON               | ON                | -    | -    | MS                          | MS                          |
| tion into MS            |                  |                    |      |      |                             |                             |
| Sample type             | CSF(9), serum(5) | CSF(15), serum(10) | serum | CSF | CSF(10), serum(10)        | CSF(10), serum(10) |
| Gender (female/male)    | 3/6              | 13/3              | 7/20 | 8/3  | 7/3                        | 9/1                        |
| Age (average)           | 35               | 32                | 64   | 31   | 32                          | 32                          |

Serum = serum or plasma.
* 1 sample with non-native Finnish background.
** 2 samples with non-native Finnish background.
out of which 350,000 peptides, on average, were with unique amino acid sequence (data structure shown on Figure S1b). Altogether, the size of the described antigenic repertoire of the discovery cohort encompassed about 20 million unique peptide sequences (data not shown). Although the majority of these peptides were largely individual-specific as observed by the data structure analysis of the most frequent 5000 and 20,000 peptides from each sample (top 5000 and top20000 peptides, respectively), the studied samples shared a substantial fraction of common peptide antigen characteristics across all datasets (Figure S1c shows the heatmap image of MVA profiles from the CSF samples of patients in the discovery cohort).

2.4. Data processing and statistical analysis

Data processing was performed as described previously [16]. Statistical analyses (ANOVA, t-test, correlation analyses, Receiver Operating Characteristic (ROC), distribution profiles) and corresponding visualisations were done using MedCalc Statistical Software (version 17.0.4, MedCalc Software, Belgium). For clustering and hyper-geometric test SPEXS2 Software was used. In-house data analysis scripts were used to perform peptide divergence, peptide abundance, and coefficient of variation computations, as well as motif alignments and sequence annotations against user-defined reference sequence database (IEDB.org (01.08.2019)). Excel VBA (Visual Basic for Applications) scripts were used for these data visualisations.

2.5. Measurements of total IgG and protein

Total amount of IgGs in sera/plasma and CSF were measured using ELISPOT method. In brief, diluted sera/plasma samples and CSF samples were printed onto nitrocellulose film slides (Amersham Bioscience) by SpotBot® 4 Personal Microarrayer (Arrayit). Purified human IgG (Sigma, i4506) was used for standard curve preparation with rabbit anti-human IgG (HRP) (Abcam, ab6759; dilution 1:1000) as a secondary antibody. Images were scanned using Ettan™ DIGEImager (GE Healthcare Life Sciences).

2.7. Western blot analysis

For western blot (WB) analysis, recombinant phages displaying sequences of interest (ELEKAYKTLSSY (vector of cluster A), TLPMDTSPRAHW (vector of cluster B) and DYKDDDDK (FLAG tag)) at the N-terminus of the plII of the M13 were generated with in vitro mutagenesis. For WB, 30 μg of protein lysate of 1 × 10^13 phages with the following primary antibodies: anti-Flag (Sigma-Aldrich F3165; 1:3600), precleared human sera (dilution 1:750) and precleared human CSF (dilution 1:7,5) samples were incubated with secondary rabbit anti-mouse (Abcam, ab6728; dilution 1:10 000) or rabbit anti-human IgG antibodies (Abcam, ab6759; dilution 1:10 000). The ECL Femto kit (Amersham) was used for detection of target proteins.

2.8. CMV and EBV seropositivity

Human Cytomegalovirus- (CMV) and EBV-specific IgGs were determined by the ISO 17,025 accredited enzyme-linked immunosorbent assay (ELISA). For CMV, for analysis of sera/plasma anti-CMV IgG ELISA kit (EUROIMMUN EI 2570 9601 G) was used. Anti-EBV IgG ELISA kit (EUROIMMUN EI 2731 9601) was used for measuring EBV seropositivity in sera/plasma samples. Analyses were carried out in accordance with the manufacturer’s specifications. absorbence was measured at 450 nm with SpectraMax Paradigm (Molecular Devices).

2.9. Role of the funding source

We confirm that all funders played no role in study design, data collection, data analysis, interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

3. Results

3.1. Discovery study of antibody epitope profiles differentiating ON from MS

Effective biomarkers for assessment of ON prognosis, in particular those measurable in blood, are largely lacking. MS is driven by systemic immune activation of autoimmunity against CNS, thereof CSF samples of the patients shown in Figure S1c. Analysis of the most abundant fractions resulted in defined sets of peptides that were common to samples of CSF and sera/plasma from the same individual (>0.7 (Pearson); p<0.001 (ANOVA); Fig. 1b-c). Subsets of peptides or peptide-displaying phages printed onto nitrocellulose filter pads (Amersham Bioscience) by SpotBot® 4 (Arrayit) were exposed to human precleared sera/plasma (dilution 1:100) or CSF (dilution 1:2) for 1 h at room temperature (RT) and then incubated with rabbit anti-human IgG (HRP) (Abcam, ab6759; dilution 1:1000) as a secondary antibody. Images were scanned using Ettan™ DIGEImager (GE Healthcare Life Sciences).
3.2 Two immunodominant epitopes discriminate between ONON and ONMS

To examine the group-discriminating features, using unsupervised clustering of the most abundant (immunodominant) peptides from each study cohort (altogether 1.4 million peptides) we delineated 1669 group-specific epitopes (p<0.005 (hypergeometric test)). The overall data analysis scheme is shown in Figure S2a. Figure S2b shows the distribution of these 1669 epitopes across study cohorts. Correlation analysis that followed revealed two major consensus motifs, clusters A and B (coefficient of variation >1.2, Figure 2a) and top sequence motifs are listed in Table S2). Cluster A contained peptides with Y.TLY amino acid patterns, whereas cluster B was formed by P...T.PR pattern-containing peptides. The heatmap analysis of median peptide abundance as shown in Figure 2b further confirmed the differential distribution of peptides forming clusters A and B in different clinical cohorts.

Next, we evaluated the association of clusters A and B with clinical diagnosis using ANOVA. In blood samples, as shown in Figs. 2c, cluster A epitopes were most abundant in ONON and controls (p<0.05 (ANOVA) as compared to ONMS), whereas a trend of cluster B epitopes being more abundant in ONON and ONMS as compared with controls was observed (p = 0.077 (ANOVA)). Furthermore, the group-discriminating features of clusters A and B were similarly detected in the CSF of ONON, ONMS, and ON other as compared with controls (cluster A CTRL vs. ONON p<0.005 (ANOVA); cluster B CTRL vs. ONON p<0.005 (ANOVA); cluster A CTRL vs. ONMS p<0.05 (ANOVA); cluster B CTRL vs. ONMS p<0.05 (ANOVA); Figure 2c).

3.3 Immunodominant epitopes A and B mimic highly antigenic epitopes of CMV and EBV

Epitopes of clusters A and B harvested the most abundant antibody immune response, suggesting their possible association with common human pathogens. Reviewed epitopes of human pathogens from IEDB database were aligned to consensus sequences of clusters A and B. Peptides of cluster A unequivocally defined the antigen domain 2 (AD2) of the neutralising epitope of CMV glycoprotein B (gB), whereas peptides of cluster B were close mimics of the C-terminal epitope of capsid antigen (VCA) p18 protein of EBV (Figure 3). As shown in Figure 3a and 3b and further corroborated by western blot analysis by using recombinant phages displaying prototype peptides, epitopes derived of AD2 (TTNLYN<sub>20</sub>) of CMV gB and of EBV VCA p18 (GGQPHTAPS<sub>20</sub>) could act as true antigens mimicked by clusters, i.e., epitopes of A and B, respectively. Further validation using ELISPOT analyses confirmed the MVA detected seropositivity of epitopes A and B at accuracy of 95%. Comparative analysis of seropositivity by MVA and ELISPOT are shown in Figure S3. The findings of the herpesviral antigenic background of these two epitopes were further strengthened by clinical ELISA testing. The highest anti-CMV seroresponse was observed in the cohorts of ONON, MS other and CTRL, whereas all study subjects in the discovery cohort were seropositive for anti-VCA EBV (percentages of CMV and EBV seropositive and -negative subjects in study groups are shown in Figure S3b). Moreover, all study subjects, who showed antibodies against clusters A or B epitopes in MVA, were seropositive for CMV and EBV, accordingly (Figure S3c and S3d).

Interestingly, the alignment analysis of other MVA-defined consensus motifs (Figure 2a) resulted in delineating epitopes associated with Epstein-Barr nuclear antigen 1 (EBNA1) and also with other
epitopes of gB of CMV (Table S3). However, these were detected by MVA at considerably (50 to 135 times) lower abundances as compared to epitopes of A and B. Nonetheless, the epitopes aligning to EBNA1 were clearly more frequent in ONMS as compared to controls (Table S3).

3.4. Value of epitopes A and B as clinical biomarkers

We next tested epitopes of clusters A and B in the independent validation cohort of 20 subjects with relapsing MS and in 448 controls (Fig. 4 and Tables 1 and 2). MVA analysis of the blood samples of the validation cohort showed that MS patients either with ON onset (MSON) or other MS onset (MSother) had roughly similar patterns of response to peptide epitopes of A and B, although these of MSON differed slightly more from the controls (Figure S4a). Fig. 4a shows that by combining sera findings from ONMS and MSON groups, clusters A and B epitopes specifically discriminated between these diagnostic groups and controls (p<0.05 (ANOVA), Fig. 4a). Furthermore, normalising the ELISPOT findings from CSF of MS patients to total IgG content yielded a high positive correlation between CSF and peripheral blood in the immunoreactivity to epitopes of A and B (r = 0.95 (Pearson), p<0.0001 (t-test); r = 0.71 (Pearson), p<0.0001 (t-test); Fig. 4b). The measurements of total amounts of proteins and IgGs in the CSF samples of study cohorts are in Figure S4b.

We used ROC analysis to assess the predictive value of epitopes of clusters A and B as blood-based diagnostic biomarkers for MS after ON (Fig. 4c and S4c). The prediction value of these two epitope biomarkers was high in stratifying subjects with ON MS and MSON (n = 20) from healthy controls (n = 256) (Table 1 and 2) with balanced accuracy of 74.61 at 75% sensitivity and 74.22% specificity (AUC 0.796,
CI 0.743 to 0.842, \( p < 0.0001; \) Fig. 4c). The ROC curve analysis is shown in Fig S4c, and the calculated prediction values for each patient are shown in Table S4. Moreover, when the independent external cohort of subjects with non-demyelinating disease (\( n = 192; \) Table 2) was used as a control group, the prediction values for clusters A and B epitope biomarkers for blood-based detection of MS with ON onset were even higher with balanced accuracy of 76.62 at 60% sensitivity and 93.23% specificity (AUC 0.82, CI 0.761 to 0.869, \( p < 0.0001; \) Fig. 4c and S4c, Table S4).

### 4. Discussion

In this study, we present an unbiased antibody epitope discovery strategy that resulted in delineation of two epitope biomarkers stratifying subjects with non-demyelinating disease from those with MS after ON from healthy controls and patients without any demyelinating disease. Using hypothesis-free MVA immunoprofiling approach we observed highly individual immune response profiles in the paired peripheral blood and CSF samples of the study subjects (Fig. 1). Group comparative

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Epitope clusters A and B mimic highly antigenic epitopes of CMV and EBV. a-b) Heatmap images of epitopes of clusters A and B alignments to IEDB epitopes (01.08.2019). The criteria for homology searches were set to 4 amino acid similarity matches and group median values for each epitope motif were used in alignment calculations. On top of the heatmap panels primary sequences of gB CMV (Uniprot code Q2FAM8) and VCA p18 EBV (Uniprot code P14348) are shown with amino acids defining the core epitopes of clusters A and B. Below the heatmap panels, the scale of relative alignment loads (0–35 000) is shown in colour code. The acronyms of the discovery and validation cohorts are shown in the left. Average_rnd — average median values of scrambled motifs derived of clusters A and B motifs. Representative images from validation studies of antigenic epitope predictions using western blot analysis are shown in the lower part of the figure (see full blots on Figure S5). Western blot analysis of recombinant phages containing the epitope gB CMV or VCA EBV was performed using recombinant phages encoding Y.TLY-pII or P.T.PR-pII (1) and flag-pII fusion proteins (2) with primary antibodies: i) pre-cleared serum/plasma (dilution 1:750), CSF (dilution 1:7.5); ii) mouse anti-FLAG antibody (Sigma Aldrich, No. 287) and secondary antibodies: i) rabbit anti-human-HRP (Abcam), ii) rabbit anti-mouse-HRP (Abcam). Protein molecular weight markers (kDa) are shown in the left. Gel staining — protein loading control with Coomassie blue staining, pIII/pIII protein of M13. Source of primary antibodies is indicated on top of the blot.

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Epitope clusters A and B predict MS with ON onset at high diagnostic accuracy in validation cohorts. a) Validation of epitopes of clusters A and B by MVA confirmed the diagnostic power of these as two blood-based biomarkers to detect MS with ON onset. Box plot analysis of immunoreactivity values of clusters A and B epitopes as obtained by MVA in sera samples of MS with ON onset (samples of ONMS, \( n = 20 \)) and controls. y-axes - peptide abundance (in log10); x-axes - study cohorts; \( p \) - ANOVA on log-transformed data \( p \) value, * - \( p < 0.05; \) ** - \( p < 0.001; \) *** - \( p < 0.0001. \) b) High immunoreactivity to epitopes of clusters A and B in sera was always co-detected with high immunoreactivity in CSF. Pearson correlation analysis of ELISPOT values of gB CMV (cluster A) and VCA p18 of EBV (cluster B) in the CSF samples of MS and MS other patients (\( n = 20 \)) upon normalisation to total IgG amount. y-axes - ELISPOT values in CSF; x-axes - ELISPOT values in serum; \( r \) - correlation coefficient; \( p \) - correlation significance level (t-Test). c) ROC analysis data of the predictive value of clusters A and B epitope biomarkers in different validation groups — MS with ON onset (samples of ONMS, \( n = 20; \) Table 1-2), healthy (CTRL samples from discovery cohort \( n = 27 \)) and samples from external cohorts of healthy individuals (\( n = 256 \)) and subjects with non-demyelinating diseases (\( n = 192; \) Table 2). Accuracy — balanced accuracy of sensitivity and specificity; area under the curve (AUC).
clustering analysis of peptide epitope profiles identified case-discriminating antigenic repertoires forming two major clusters A and B (Fig. 2). Our further analysis determined these epitopes A and B as mimics of highly antigenic epitopes of CMV and EBV (Fig. 3). In validation analyses, we confirmed these two epitopes (A and B) as highly potential serologic biomarkers of MS (Fig. 4).

Antibody epitope repertoire shared similarity in the CSF and serum. Our study demonstrated the power of next generation phage display MVA method for high-throughput profiling of antibody response at the epitope precision of the clinical samples from the early phase of MS with ON onset. Despite the highly individual top immune response profiles, we observed that paired sera and CSF samples showed highly similar antigenic features (Fig. 1), arguing for shared robust antibody responses both in the periphery and in the CNS. Although findings of similar patterns of immunoreactivity to single antigens in the peripheral blood and CSF have been reported (29–31), our current data extended these findings to the depths of millions of peptide antigens. Furthermore, amongst these peptide antigens we describe repertoires (epitopes forming clusters A and B) that can distinguish different demyelinating inflammatory conditions (ONON, ONMS, MSON and MSother) and controls (that can distinguish different demyelinating antigens we describe repertoires (epitopes forming clusters A and B) millions of peptide antigens. Furthermore, amongst these peptide homogeneity of the studied population. In addition, this is the only the two major and highly antigenic epitopes could be thoroughly studied. It also remains unclear how these associations between sera and CSF profiles of epitopes A and B relate to MS pathogenesis and whether these associations are cause or consequence of the disease. Further delineation of detected antibody epitopes is desirable as these might provide further understanding of the pathophysiological context behind MS progression.

Our findings indicate that specific antibody epitope biomarkers of MS exist in the blood of patients at different stages of disease. These epitope biomarkers can be combined into a multivariate model with high discriminatory potential. Further validation of the selected two-epitope biomarker analysis from this initial study are warranted to assess their exact value in MS development. Overall our results stress the importance of dissecting global antibody immunoreactivity patterns at the epitope level towards the personalised care of MS.

Contributors

HS - conceptualisation, data processing, formal analysis, investigation, methodology, software, supervision, validation, data visualisation, writing original draft, reviewing and editing; AP - data processing, formal analysis, investigation, methodology, software conceptualisation, formal analysis, visualisation; MJ - data processing, investigation, methodology, software conceptualisation, data visualisation, writing, reviewing and editing; NP - writing, reviewing and editing; AR - writing, reviewing and editing; MT - writing, reviewing and editing; AV - conceptualisation, writing, reviewing and editing; JN - clinical data analysis, writing, reviewing and editing; MS - funding acquisition, clinical data analysis, writing, reviewing and editing; PT - conceptualisation, patient recruitment, funding acquisition, clinical data analysis, writing, reviewing and editing, and KP - conceptualisation, formal analysis, investigation, methodology, visualisation, project administration, supervision, funding acquisition, writing original draft, reviewing and editing. All authors had full access to all underlying data. All authors read and approved the final version of the manuscript.

Data sharing statement

Data on annotation are freely available from the database of IEDB.org. Any data not published within the article or supplementary materials will be shared in anonymised format by request from any qualified investigator. If desired, please contact the corresponding author of this article.

Declaration of Competing Interests

AP and KP are inventors of the patent application (PCT Application No. US/14079626) filed by Protobios that covers the use of phage display method to manipulate and monitor humoral immunity. PT
reports reimbursement for activities not related to the present article. All other authors declare no competing interests.

Acknowledgements

We thank the Protobios’ team for their expertise and availability that supported this work. We are grateful to Dr. Allan-Herrmann Pool (Caltech) for the critical reading of the early versions of the manuscript. We thank Liia Jansson for excellent technical assistance. This work was supported by grants of Protobios from the Estonian Ministry of Education (5.1-4/20/17), from the Estonian Research Council PRG573 and PSS691), also partly from the EU programs of H2020-MSCA-RISE-2016 (EU734791) and H2020 PANBioRA (EU760921), Helsinki University Hospital grants, Mary and Georg C. Ehrnrooth Foundation, the Finnish Eye Foundation. KP was partially supported by grant PRG805, the Estonian Research Council. AV was supported by Sigrid Juselius Foundation and by Magnus Ehrnrooth Foundation.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103211.

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