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Autoimmunity

Autoreactive T cells identified in patients with anti-Jo1 + antisynthetase syndrome recognise a new epitope on histidyl t-RNA synthetase

Angeles S. Galindo-Feria[1,2,3], Ravi Kumar Sharma[1,2,4], Anatoly Dubnovitsky[1,2], Christina Gerstner[1,2], Genadiy Kozhukh[1], Annika Van Vollenhoven[1,2], Juan Sebastian Diaz Boada[1], Daniel Ramsköld[5], Adnane Achour[6,7], Maryam Dastmalchi[2,3], Hugh H. Reid[8], Tatyana Sandalova[6,7], Jamie Rossjohn[8,9], Karine Chemin[1,2], Vivianne Malmström[1,2], Ingrid E. Lundberg[1,2,3], Begum Horuluoglu[1,2,*]

[1] *Division of Rheumatology, Department of Medicine, Solna, Karolinska Institutet, Stockholm, Sweden*
[2] *Center for Molecular Medicine, Stockholm, Sweden*
[3] *Department of Gastro, Dermatology and Rheumatology, Karolinska University Hospital, Stockholm, Sweden*
[4] *Department of Clinical Immunology and Rheumatology, All India Institute of Medical Sciences, Bilaspur, (H.P), India 174001*
[5] *Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden*
[6] *Division of Infectious Diseases, Karolinska University Hospital, Stockholm, Sweden*
[7] *Science for Life Laboratory, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden*
[8] *Infection and Immunity Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, VIC, Australia*
[9] *Institute of Infection and Immunity, Cardiff University, School of Medicine, Heath Park, Cardiff, UK*

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ABSTRACT

Objectives: Anti-Jo1 + antisynthetase syndrome (ASyS) is characterised by autoantibodies targeting histidyl t-RNA synthetase (HisRS), association with *HLA-DRB1*03:01* and a distinct clinical phenotype including interstitial lung disease, myositis, arthritis, and mechanic's hands. Previous studies of autoreactive HisRS-specific $CD4^+$T cells point to yet undiscovered T cell epitopes. We aimed to identify new epitopes on HisRS to investigate the presence of autoreactive T cells and their corresponding T-cell receptor (TCR) repertoire from patients with ASyS.

Methods: Peptides from HisRS N-terminal region with appropriate major histocompatibility complex (MHC) anchor residues were selected for *in vitro* binding assays. The peptide ($HisRS_{41-55}$) with the highest HLA-DRB1*03:01 binding affinity was selected for studies with HLA-class II tetramers. Peripheral blood mononuclear cells (PBMCs) from patients with ASyS with *HLA-DRB1*03:01* (n = 12) were stimulated *in vitro* with peptide and peptide-HLA-DRB1*03:01 tetramers were used to detect $HisRS^+CD4^+$T cells. Single TCR sequencing of captured T cells allowed analyses of the underlying TCR repertoire.

Results: We identified a new T cell epitope on HisRS with high affinity for HLA-DRB1*03:01. Autoreactive $HisRS^+CD4^+$T cells were detected in PBMCs of patients (n = 6/12). TCR repertoire analysis of $HisRS^+CD4^+$T cells revealed shared gene V-alpha and beta usages. Moreover,

*Correspondence to Dr. Begum Horuluoglu, Karolinska Institute, Stockholm, Sweden.
E-mail address: begum.horuluoglu@ki.se (B. Horuluoglu).
Vivianne Malmström and Ingrid E. Lundberg contributed equally.
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HisRS$^+$CD4$^+$T cells persisted after treatment in 2 patients (P2 and P4) and 2 identical T cell clones were detected between the initial and follow-up time points in 1 patient (P2).
Conclusions: Autoreactive T-cells targeting a new HisRS epitope were identified indicating T cell reactivity to diverse epitopes of the HisRS protein in patients with anti-Jo1 autoantibodies. Furthermore, we demonstrated the TCR repertoire of autoreactive HisRS + CD4 + T cells in patients. Persistence of these T-cells and specific clones may be contributing to disease.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Association of *HLA-DRB1*03:01* with antisynthetase syndrome (ASyS) indicates the contribution of CD4$^+$T cells in disease.
- CD4$^+$T cells from peripheral blood mononuclear cell and bronchoalveolar lavage fluid cells from patients with ASyS are activated upon stimulation with the full-length histidyl t-RNA synthetase (HisRS) and HisRS-derived peptide indicating the presence of HisRS-reactive T cells.
- Shared T cell clones are found between muscle and blood with a cytotoxic phenotype. These clonally expanded T cells persist with a similar phenotype in a patient with ASyS despite treatment.

WHAT THIS STUDY ADDS

- A new T-cell epitope identified on HisRS that has a high affinity to bind HLA-DRB1*03:01.
- Autoreactive HisRS$^+$CD4$^+$T cells were identified using HLA-class II tetramers in 6 patients with ASyS upon *in vitro* stimulation.
- T-cell receptor (TCR) repertoire analysis of autoreactive T cells showed shared alpha or beta chain CDR3 amino acid sequences and V gene usages for both alpha and beta chains among patients with ASyS.
- Persistence of antigen-specific T cells and identical T cell clones were detected after up to 12 months with immunosuppressive treatment which might contribute to disease chronicity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Presence of autoreactive T cells could be used as a tool to follow the effects of conventional treatments.
- Identification of antigen-specific T cells has implications for future development of therapies including tolerising and targeting pathogenic TCR signatures.

INTRODUCTION

Antisynthetase syndrome (ASyS) is a subgroup of idiopathic inflammatory myopathies characterised by the presence of autoantibodies against aminoacyl t-RNA synthetases, myositis, and extramuscular features such as interstitial lung disease (ILD), and is associated with high morbidity and mortality [1–3]. The most frequent anti-tRNA synthetase autoantibodies are the anti-Jo-1 autoantibodies targeting histidyl-transfer RNA synthetase (HisRS), and there is a genetic association with *HLA-DRB1*03:01* implicating the recognition of autoantigens by CD4$^+$ T cells [4,5]. T cells have been reported at the sites of inflammation in the muscle and lungs of patients with ASyS where they are proposed to contribute to disease pathogenesis [6–8].

HisRS, which is a cytoplasmic enzyme involved in protein translation, may trigger innate and adaptive immune responses leading to cell-mediated damage in tissues such as muscle and lung [9,10]. Our group has shown that CD4$^+$T cells from peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage fluid (BALF) from patients with anti-Jo-1$^+$ ASyS with the *HLA-DRB1*03:01* haplotype were activated upon stimulation with HisRS protein. Increased levels of CD40L were detected on CD4$^+$T cells, along with enhanced interferon γ (IFNγ) production, suggesting the presence of T cells recognising the HisRS protein [11]. Interestingly, while almost all patients' T cells responded to the full-length HisRS protein, not all responded to stimulation with the peptide used for T cell stimulation ($HisRS_{11\text{-}23}$) suggesting that multiple epitopes may contribute to the T cell response. These findings highlight the potential for epitope spreading and the importance of identifying new epitopes on HisRS.

The recombination of T-cell receptor (TCR) gene segments is the basis for the generation of a diverse T cell repertoire where each T cell expresses a unique TCR sequence. Restricted TCR gene usage in the lungs and muscles of patients with myositis has been shown suggesting antigen-induced T cell responses in these organs [12]. More recently, we showed using single-cell RNA sequencing the presence of clonally expanded cytotoxic CD4$^+$T cell clones shared between muscle and blood of a patient with anti-Jo1 + ASyS suggesting the possibility to detect pathogenic T cells in blood [13]. These clonally expanded cytotoxic CD4$^+$ T cells persisted with the same phenotype in both blood and muscle after 9 months of conventional immunosuppressives treatment, further supporting the contribution of CD4$^+$T cells to disease chronicity [13].

In this study, we aimed to identify autoreactive HisRS-specific CD4$^+$T cells in the peripheral blood of patients with ASyS using HLA-class II tetramers loaded with a HisRS peptide, to detect low-frequency antigen-specific CD4$^+$T cells with high efficiency. We further performed single TCR sequencing to study the TCR repertoire of autoreactive HisRS-specific T cells and investigated their persistence at different disease stages.

METHODS

Patients and healthy controls

Peripheral blood samples from 2 cohorts (n = 33 in total) with anti-Jo1 autoantibodies were collected from Karolinska University Hospital Rheumatology Clinic at the time of diagnosis and at annual follow-up between 2014 and 2023. The diagnosis of ASyS was based on the presence of anti-Jo1 autoantibodies in addition to one of the following features: ILD, myositis, arthritis, Raynaud's phenomenon, fever, or mechanic's hands [14]. First cohort consisted of 19 patients who were included in peptide stimulation assays, whereas the second cohort with 14 patients was included for the HLA-class II tetramer assays. Disease activity was assessed using the International Myositis Assessment and Clinical Studies core set measures, including the Myositis Disease Activity Assesment Visual Analogue Scale (MYOACT), manual muscle testing 8 (MMT8), and the physician global assessment. A detailed, comprehensive summary of demographic, clinical, laboratory, immunological treatment, and genetics for both cohorts can be found in the supplementary methods and Supplementary Tables S1 and S2. HLA-

DRB1 alleles were genotyped using the DR low Olerup SSP kits. Peripheral blood cells from *HLA-DRB1*03*-positive healthy controls were kindly provided by the Uppsala Bioresource, Uppsala University Hospital.

Ethics

The research ethics committees at Region Stockholm (DNR: 2005/792-31/4, 2018/1198-32) and Uppsala University (DNR: 2009/013) approved the study. Written informed consent was obtained from all participants. This study was conducted in accordance with the Declaration of Helsinki.

RESULTS

Antigen-specific CD4$^+$ T cells are detected using in-house produced and assembled peptide-HLA-class II tetramers

First, we evaluated the efficacy of our tetramer assay to detect antigen-specific CD4$^+$T cells *in vitro* using HLA-DRB1*03:01 tetramers (Tmr hereafter) loaded with the positive control tetanus peptide ($Tet_{506-525}$). PBMCs from HLA-DRB1*03:01 positive healthy donors (HC1, HC2, and HC3) were stimulated with $Tet_{506-525}$ along with interleukin(IL)7 and IL15 cytokines to support T cell survival (Fig 1A). The cells were kept in culture for up to 21 days and stained for the presence of tetanus-reactive T cells on different days. Tetanus-HLA-DRB1*03:01 Tmrs conjugated with 2 different fluorophores were used to gate on double Tmr-positive T cells in order to minimise false positive events from the cultured cells using flow cytometry (Supplementary Fig S1A). We observed that the number of tetanus-specific CD4$^+$T cells increased with the number of incubation days and decided to continue with day 13 cultures for the detection of antigen-specific CD4$^+$T cells (Fig 1B,C). To further confirm the presence of $Tet_{506-525}{}^+$CD4$^+$T cells, we measured IFNγ secretion following stimulation of PBMC from 2 individuals (HC 1 and patient with ASyS). $Tet_{506-525}$ Tmr$^+$ cells were detected in HC1, while no Tmr$^+$ cells were detected in the patient (Fig 1D). FluoroSpot results showed a significantly higher number of IFNγ^+ cells (spots) in culture containing tetanus Tmr-positive CD4$^+$T cells as compared to their unstimulated counterparts. No such increase in the IFNγ^+ cells was observed in the cultures without tetanus Tmr-positive cells (Fig 1E). Altogether, our results indicate that our experimental set-up enabled us to detect

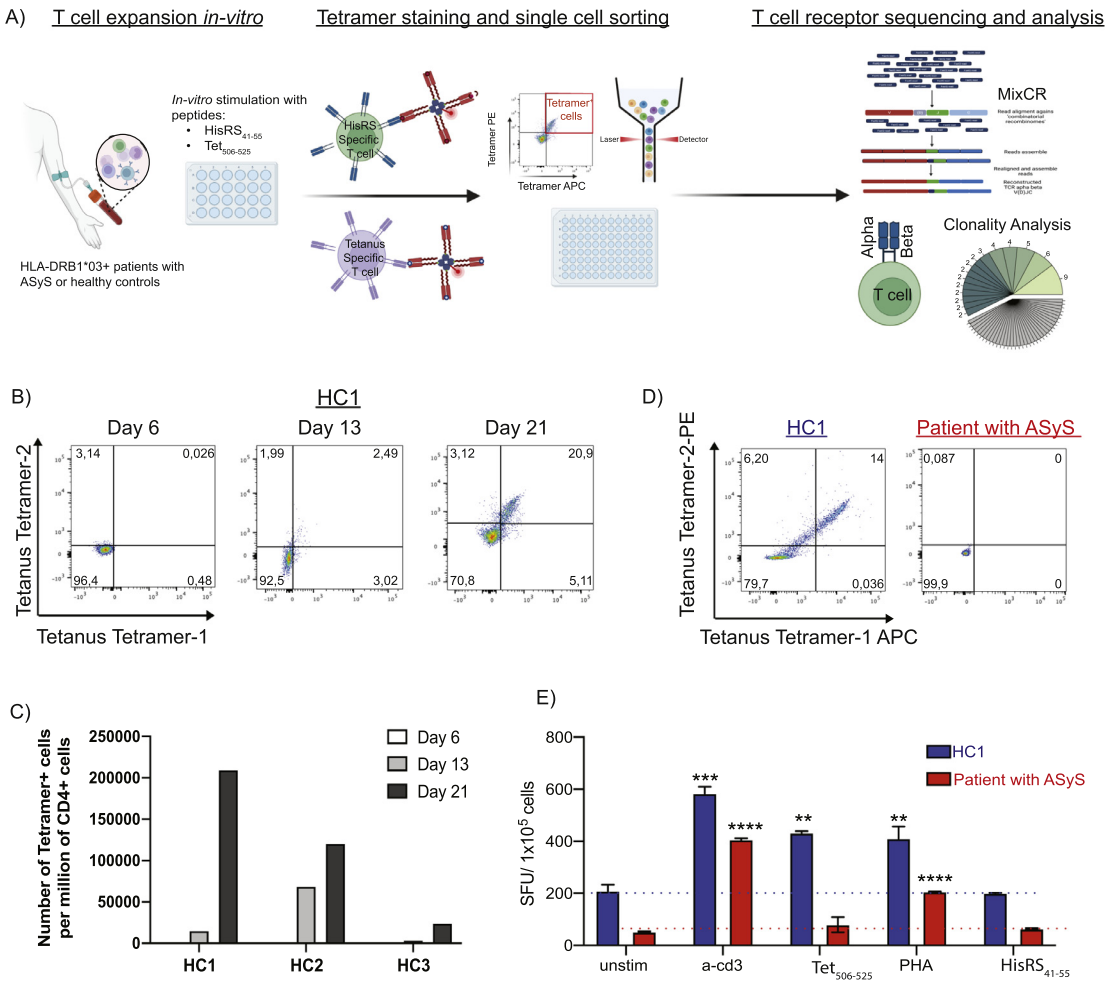

Figure 1. Tetanus tetramer staining of antigen-specific CD4$^+$T cells from human peripheral blood samples (A) Pipeline workflow starting from expansion of human peripheral blood mononuclear cells with respective peptides ($HisRS_{41-55}$ and $Tet_{506-525}$) along with IL7 and IL15, Tmr staining, single T-cell sorting and sequencing. (B) Representative flow plots showing tetanus-specific cells using $Tet_{506-525}$ tetramers conjugated with 2 different fluorophores on days 6, 13, and 21 following stimulation with $Tet_{506-525}$ from healthy control 1 (HC1). (C) Bar plot showing the number of $Tet_{506-525}$ Tmr$^+$ CD4$^+$T cells per million of CD4$^+$T cells on days 6, 13, and 21 from 3 healthy control (HC1, HC2, and HC3) peripheral blood sample following stimulation with $Tet_{506-525}$. (D) Flow plots showing presence (HC1, blue) and absence (patient with ASyS, red) of tetanus Tmr-positive CD4$^+$T cells, and (E) fluorospot staining from cells of these individuals which are positive for IFNγ upon stimulation with $Tet_{506-525}$ or a-CD3 or PHA as positive controls and $HisRS_{41-55}$ and unstimulated as negative controls. Number of spots per well was normalised to spots in 100,000 cells/well for plotting purposes. ASyS, antisynthetase syndrome; IFNγ, interferon γ; IL, interleukin; TMR, tetramer; HisRS, histidyl t-RNA synthetase; PHA, phytohemagglutinin.

antigen-specific T cells using in-house assembled peptide-HLA-DRB1*03:01 tetramers.

*A new T cell epitope on HisRS identified and loaded on peptide-HLA-DRB1*03:01 tetramer*

Given the known binding preferences for peptides to HLA-DRB1*03:01 and the previous reports on HisRS T cell reactivity being confined to the granzyme cleaved part of HisRS, we predicted tentative T cell epitopes for testing their binding to HLA-DRB1*03:01 [11]. A new epitope $HisRS_{41-55}$ was identified (LKAQLGPDESKQKFV, predicted binding residues underlined) with a higher binding affinity to HLA-DRB1*03:01 compared to the previously reported epitope $HisRS_{11-23}$ (Fig 2A). Binding assays comparing the $HisRS_{41-55}$ and $HisRS_{41-55}$-mut peptides (LKAALGPDASKAKFV, mutations underlined) showed no difference in HLA-binding, supporting that the identified anchor residues are indeed responsible for the interaction (Fig 2A). We also compared the stimulatory effects of these peptides by incubating PBMCs from patients with ASyS (n = 19, Supplementary Table S1). An increased CD40L expression was detected on $CD4^+$ T cells in 53% of the patients stimulated with $HisRS_{41-55}$, whereas 32% of patients had an increase upon $HisRS_{11-23}$ stimulation compared to unstimulated (Supplementary Fig S1B,C). There was no significant difference in CD40L upregulation between peptide stimulations (Supplementary Fig S1C). Thus, the new epitope identified ($HisRS_{41-55}$) was chosen for the assembly of peptide-HLA-DRB1*03:01 tetramers.

Autoreactive HisRS-specific $CD4^+$ T cells are detected in PBMC of patients with anti-$Jo1^+$ ASyS upon in vitro expansion

Next, we stimulated PBMCs from patients with anti-$Jo1^+$ ASyS (n = 12/14 with an *HLA-DRB1*03* haplotype, Supplementary Table S2) with $HisRS_{41-55}$ and $Tet_{506-525}$ [15] peptides, to investigate the presence of antigen-specific T cells using tetramers. We included antibodies against cell surface receptors previously described to be upregulated on antigen-specific T cells (CD69, CD25, PD1, and CD137) [16,17] and a cytotoxicity-related receptor (GPR56) [18]. Between days 10 to 14 of stimulation, we detected HisRS Tmr double ($Live^+CD3^+CD4^+$ Tmr-APC^+/Tmr-PE^+) positive T cells in 6 of 12 patients, and

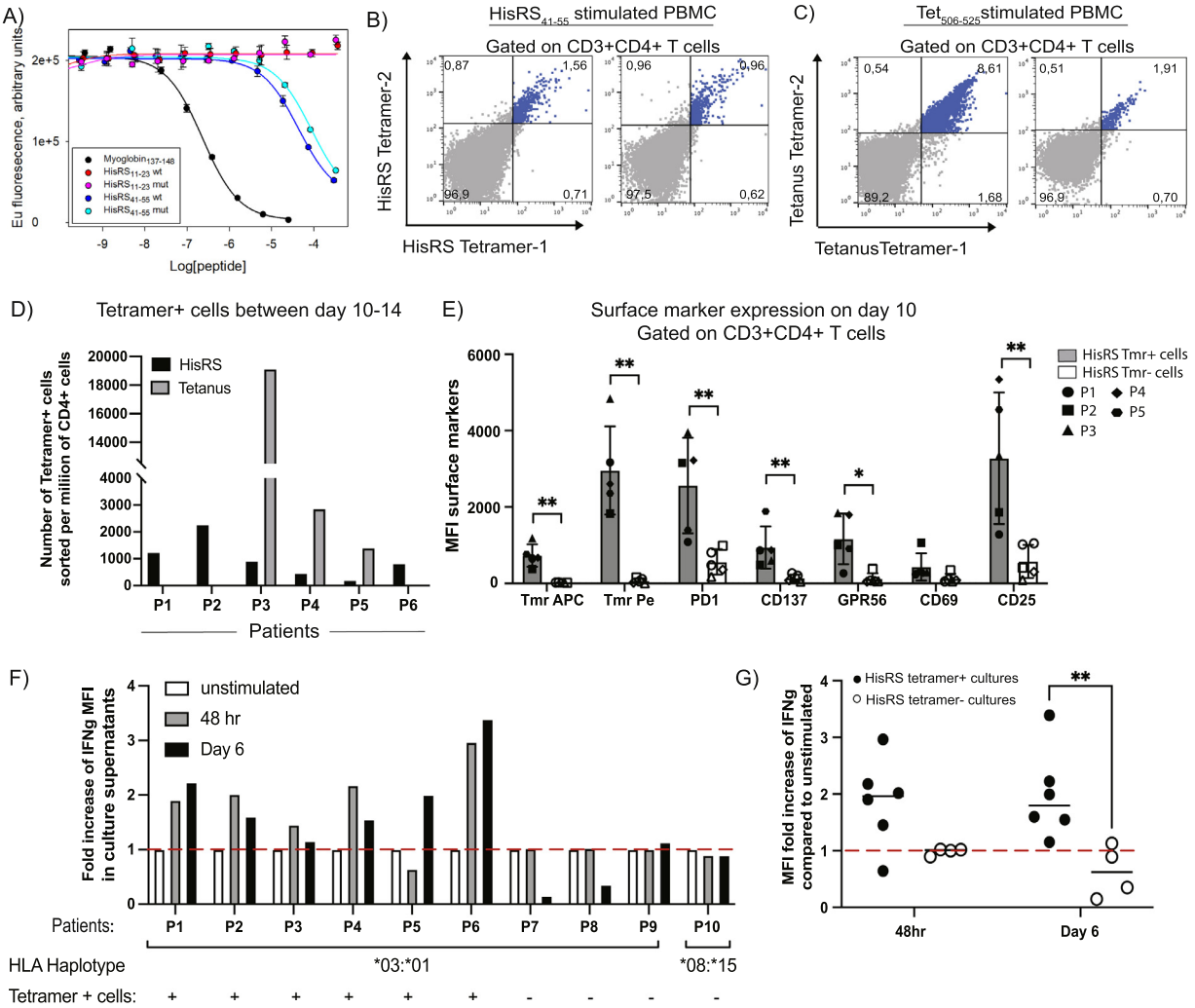

Figure 2. HisRS-specific $CD4^+$ T cells detected in peripheral blood of patients with ASyS. (A) Binding of HisRS peptides $HisRS_{10-25}$ (red), $HisRS_{10-25}$ mutated (pink), $HisRS_{41-55}$ (blue), $HisRS_{41-55}$ mutated (light blue) and myoglobulin (black) to MHC II. Representative flow cytometry plots showing (B) HisRS Tmr and (C) tetanus Tmr-positive cells (blue) among all $CD4^+$ T cells (grey) upon stimulation with $HisRS_{41-55}$ and $Tet_{506-525}$, respectively, using patient PBMCs. (D) Number of HisRS and tetanus Tmr-positive $CD4^+$ T cells per million of $CD4^+$ T cells in 6 patients P1-P6. (E) MFI of activation markers (PD1, CD69, CD137, and GPR56) from HisRS Tmr-positive (grey bar) vs HisRS Tmr-negative (clear bar) from 5 patients. Fold increase of IFNγ levels measured in supernatants of cultures at 48 h and 6 d after stimulation with $HisRS_{41-55}$ shown (F) per patient and (G) grouped based on presence of Tmr-positive cells. ASyS, antisynthease syndrome; HisRS, histidyl t-RNA synthetase; MFI, mean fluorescent intensity; P1-P6, patients 1 to 6; PBMCs, peripheral blood mononuclear cells; TMR, tetramer; MHC, major histocompatibility complex.

tetanus double Tmr-positive (Live CD3$^+$CD4$^+$ Tmr-APC$^+$/Tmr-PE) T cells in 3 of 12 patients (Fig 2B,C, Supplementary Fig S1A). There was no difference in clinical parameters in patients with detectable HisRS-specific CD4$^+$T and those without (Supplementary Table S3). The median number of HisRS Tmr-positive cells per million CD4$^+$T cells was 1044 (IQR 366-2087) in 6 patients most likely reflecting the number of HisRS-specific T cells among the seeded PBMC. We detected a higher number of Tet-Tmr$^+$ cells from the Tet$_{506\text{-}525}$ stimulated cultures compared to HisRS-Tmr$^+$ cells from HisRS$_{41\text{-}55}$ stimulated cultures due to a possible higher number of circulating tetanus-specific CD4$^+$ T cells in vaccinated adults (Fig 2D). Furthermore, HisRS Tmr-positive cells displayed significantly higher mean fluorescent intensity (MFI) of PD1, CD137, GPR56, and CD25 supporting their activation with HisRS$_{41\text{-}55}$ peptide stimulation as compared to HisRS Tmr-negative cells (Fig 2E). In the cultures from 48 hours and 6 days where HisRS Tmr-positive T cells were observed (6 patients; P1-P6), IFNγ levels were increased as compared to their unstimulated cultures (Fig 2F). Moreover, IFNγ levels were significantly higher in HisRS Tmr-positive cultures compared to Tmr-negative cultures on day 6, again supporting the antigen-specific T cell activation by HisRS$_{41\text{-}55}$ peptide in the Tmr-positive cultures (Fig 2G). Among other cytokines that were measured (IL6, IL10, tumour necrosis factor α [TNFα], and IL17A) in the supernatants TNFα levels were also significantly higher in tetramer-positive cultures at day 6 (Supplementary Fig S1D). Altogether, our data show that HisRS-specific CD4$^+$T cells are detectable in PBMC of patients with anti-Jo1$^+$ ASyS upon *in vitro* expansion.

TCR repertoire analysis revealed shared gene usages among patients

Single HisRS- and tetanus-specific CD4$^+$T cells were sorted between days 12 and 14 of peptide stimulation cultures for TCR sequencing. TCR alpha/ beta (α/β) chains of HisRS-specific CD4$^+$T cells (n = 520 T cells) and tetanus-specific CD4$^+$T cells (n = 130 T cells) were sequenced from 6 and 2 patients, respectively. T cells sharing identical complementarity determining region 3 (CDR3) amino acid sequences on both the α and β chains were considered to be a clone. Clonality analysis revealed the presence of expanded T cell clones in 5 of 6 patients for HisRS and 2 patients for tetanus (Fig 3A, Supplementary Fig S1E). The MFI values for tetramer staining of expanded T cells were confirmed from the index sorting (Fig 3B). Interestingly, we detected shared single α or β chain CDR3 sequences among different patients (Supplementary Fig S2A,B). We did not identify any clone (both α and β chain CDR3 identical) with public TCR sequences among all the tested patients with anti-Jo1$^+$ ASyS (Supplementary Table S4A). We used TCR_explore, a web-tool to further perform a TCR repertoire analysis to investigate shared patterns among TCR sequences between patients [19]. There was no difference in CDR3 lengths between patients (P1-P6) or antigens (HisRS vs Tetanus) (Supplementary Fig S2C,D). We observed a slightly higher diversity in CDR3 sequences of HisRS Tmr$^+$ cells compared to Tetanus Tmr$^+$ cells, although this difference did not reach any significance (Supplementary Fig S2E). Next, we evaluated the variable (V) and joining (J) gene pairs for both α and β chains (Fig 3C,D). We found shared gene pair usages for both α and β chains between patients for both HisRS and tetanus (Fig 3C-F, Supplementary Fig S2D,E). In addition, alpha chain V gene usages varied among individuals with AV13-1 and AV12-2 being in the top 3 gene usages observed in patients P1, P3, P5 and patients P2, P4, P5, P6, respectively, among HisRS$^+$ CD4$^+$T cells (Fig 3C, E, Supplementary Fig S2F, Supplementary Table S4B). Beta chain V gene usages showed a bias against BV20-1 among HisRS$^+$ CD4$^+$T cells which was shared between patients P1, P2, P5, and P6 (Fig 3D,F, Supplementary Fig S2G, Supplementary Table S4B). Among the tetanus$^+$CD4$^+$T cells, there were 3 CDR3α and one CDR3β sequences shared among the 2 patients. In addition, there were also 3 AVJ and 2 BVJ gene usage pairings shared between these patients (Supplementary Fig S3A-D). Thus, our results identified expanded T cell clones among HisRS$^+$ CD4$^+$T cells further supporting their specificity against HisRS$_{41\text{-}55}$ with shared alpha and beta VJ gene pairs with high diversity among HisRS-specific CD4$^+$T cells.

Persisting HisRS-specific T cells are detected in the blood of patients despite treatment

We investigated the presence of HisRS-specific CD4$^+$T cells among PBMC from follow-up samples for 4 available patients (Supplementary Table S3). In follow-up blood samples after up to 1-year with immunosuppressive treatment, we detected HisRS Tmr-positive cells upon stimulation with HisRS$_{41\text{-}55}$ peptide in 2 of these patients (P2 and P4) (Fig 4A,B). The number of HisRS Tmr$^+$ T cells was lower in the 1-year follow-up samples, compared to the samples from the first time point for both patients (Fig 4C). Similarly to the first time point, the MFI of surface markers PD1, CD137, GPR56, and CD69 as well as the IFNγ levels in the cultures were increased in HisRS Tmr-positive cells compared to the Tmr-negative cells in both patients, although this did not reach statistical significance (Fig 4D,E). Upon TCR repertoire analysis of the HisRS Tmr-positive T cells, we observed expanded T cell clones in both patients (Fig 4F). Interestingly, we detected 2 identical T cell clones between the first and second time point in patient P2 (Fig 4G,H, Supplementary Fig S3E). These data suggest that HisRS-specific T cells persist in the blood of patients despite treatment with conventional immunosuppressants. The persistence of autoreactive TCR clones may suggest their role in disease progression.

DISCUSSION

In this study, we identified a new T cell epitope on the HisRS protein with a high binding affinity to the HLA-DRB1*03:01. Autoreactive T cells against this epitope were detected using peptide-HLA tetramers in blood samples of 6 of 12 patients with *HLA-DRB1*03* and anti-Jo1$^+$ ASyS upon *in vitro* peptide stimulation. TCR repertoire analysis of HisRS-specific T cells demonstrated the presence of expanded T cell clones supporting their specificity for this epitope. No public clones were detected (sharing both alpha and beta chain CDR3 sequences) between patients indicating the diversity of the TCR repertoire. Further analysis of TCR repertoire revealed shared alpha or beta CDR3 amino acid sequences as well as variable (V) and joining (J) gene pairings for both alpha (α) and beta (β) chains among patients. Finally, we observed persisting HisRS-specific T cells in 2 of 3 patients in follow-up samples (7-16 months) despite conventional immunosuppressive treatment. Overall, our study confirms the presence of autoreactive T cells targeting HisRS-derived peptide in anti-Jo1$^+$ patients and their persistence suggests that they may be contributing to disease chronicity.

Peptide-HLA multimer techniques have identified antigen-specific CD4$^+$T cells in several autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes, systemic lupus erythematosus, celiac disease, Parkinson's disease, and

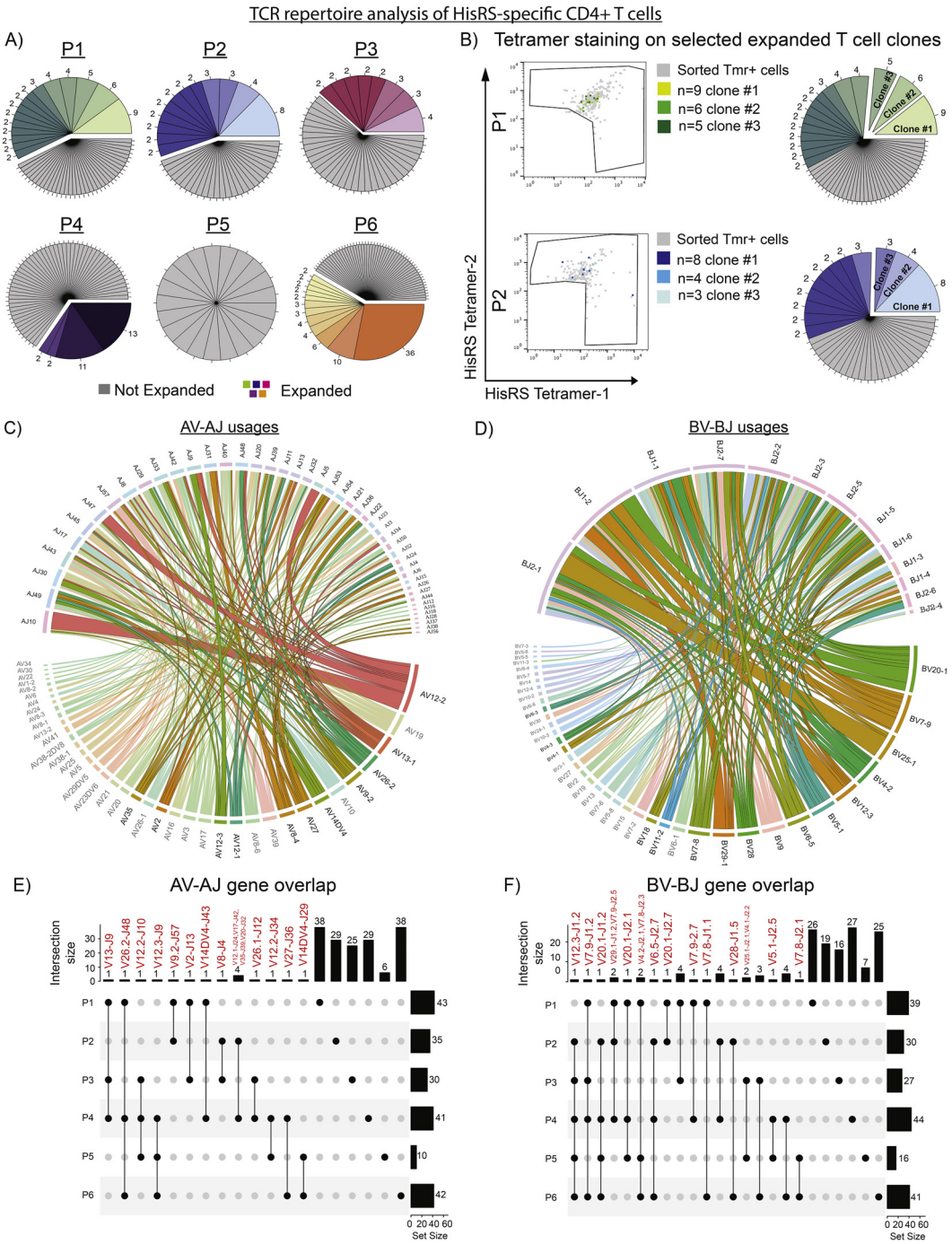

Figure 3. TCR sequencing analysis reveals expanded clones and shared VJ gene usages. (A) Pie charts showing expanded T cell clones after stimulation with $HisRS_{41-55}$ sorted with tetramers between days 12 and 14. A clone is defined as having an identical alpha and beta CDR3 amino acid sequence. Colourful slices (green, blue, pink, purple, and orange) indicate expanded CDR3 sequences, whereas grey shows unique CDR3 sequences. The number at slices corresponds to how many times a CDR3 sequence is repeated, not shown for unique sequences which is 1 for all grey slices. (B) Representative flow cytometry plots showing top 3 expanded clones from P1 and P2, for HisRS Tmr-APC and HisRS-Tmr PE. Colourful dots are corresponding to expanded T cells; grey dots are all $CD4^+Tmr^+$ cells sorted. Circos plots showing (C) alpha and (D) beta chain VJ gene pairings, each sequence is represented once. The connecting lines that have black borders are used by multiple patients. UpSet plots showing the shared gene pairings for (E) alpha and (F) beta chain. Connected dots correspond to shared usages between patients. Intersection size corresponds to how many different pairings are shared and set size corresponds to the total number of VJ pairs present in each patient. HisRS, histidyl t-RNA synthetase; MFI, mean fluorescent intensity; P1-P6, patients 1 to 6; TCR, T-cell receptor; TMR, tetramer, V, variable; J, joining.

atherosclerosis [20–28]. Presence of these T cells has been linked to disease progression, supporting their contribution to disease [25]. However, despite these advances, the α/β TCR identity of autoreactive $CD4^+$T cells remains uncharacterised in many autoimmune diseases, including ASyS. Previously, we reported the presence of antigen-reactive T cells in PBMC and BALF cells against the full-length HisRS protein in patients with anti-Jo1$^+$ ASyS. The reactivity of $CD4^+$T cells against the previously reported epitope $HisRS_{11-23}$ was limited to a subset of the patients suggesting the presence of additional immunogenic HisRS epitopes or due to epitope spreading [11]. Here, we identified a new epitope that has a higher binding affinity to HLADR1*03:01 than the previously described epitope and demonstrated the presence of

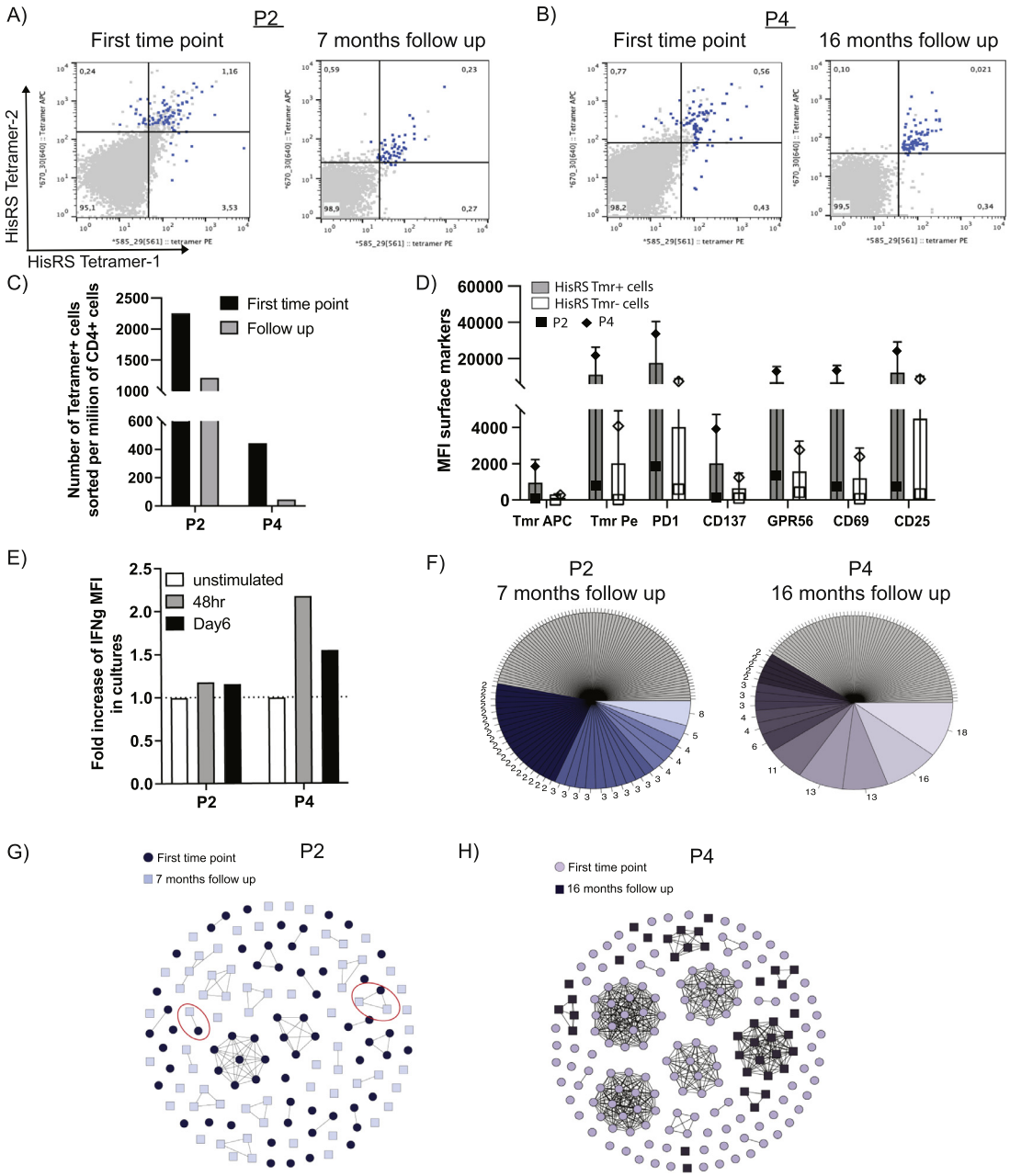

Figure 4. HisRS-specific CD4$^+$T cells detected in 1-y follow-up samples of two patients. Flow cytometry plots showing HisRS Tmr-positive cells (blue) among CD4$^+$T cells (grey) in (A) P2 and (B) P4 left panel first time point, right panel 1-year follow-up sample. (C) Number of HisRS-Tmr-postive CD4$^+$T cells per million of CD4$^+$T cells first time point (black bar) and follow up (grey bar) for two patients. (D) MFI of activation markers (PD1, CD69, CD137, and GPR56) of HisRS Tmr-positive (grey) vs HisRS tetramer negative (clear) cells (square: P2, diamond: P4). (E) Fold increase of IFNγ levels measured in supernatants of cultures 48 h and 6 d after stimulation with HisRS$_{41\text{-}55}$ shown per patient compared to unstimulated (clear bars) condition. (F) Pie charts showing expanded CDR3 sequences (expanded clones: blue for P2, purple for P4, unique clones: grey), the numbers on the slices indicate the number of cells that have corresponding sequences. Network plot showing shared CDR3 sequences between first time point (dark blue circle or dark purple circle) and 1-y follow-up (light blue square or lilac square) for (G) P2 and (H) P4. Connected shapes mean shared CDR3 sequences. IFNγ, interferon γ; HisRS, histidyl t-RNA synthetase; MFI, mean fluorescent intensity; P2-P4, patients 2 and 4; TMR, tetramer.

autoreactive CD4$^+$T cells targeting this epitope in 6 of 12 HLA-DRB1*03:01-positive patients with ASyS.

In our study, the TCR repertoire we report reflects both antigen-experienced HisRS-specific T cells as well as naïve T cells capable of responding to the HisRS$_{41\text{-}55}$ epitope, both very relevant to understand the pathology of the disease. Heterogeneity within the naïve T cell compartment has been described with certain subsets more prone to respond to specific antigens [29,30]. In support of this, Gerstner et al [28] showed that a clear proportion of the autoreactive T cells detected in patients with rheumatoid arthritis displayed a naïve phenotype, and these cells are likely to be activated *in vivo* due to high chronicity in autoimmune diseases. Taken together, this suggests that naïve T cells that can respond to autoantigens may be one of the target populations for tolerance therapies for patients with autoimmunity.

Previously, shared variable TCR gene usages in lung and muscle of patients with ASyS have been reported for selected V genes, suggesting a connection in the development and similar antigen-recognition of these cells [12]. More recently, using single-cell sequencing, we showed that cytotoxic CD4$^+$T cell clones were shared between blood and muscle in a patient with anti-Jo1$^+$ ASyS further supporting their developmental connection leading to tissue damage and demonstrating that these

potentially pathogenic T cells can be detected in peripheral blood [13].

Our TCR repertoire analysis of autoreactive HisRS-specific T cells from blood showed shared single alpha or beta CDR3 sequences among individuals, but no public clones (alpha and beta CDR3 sequences shared). However, we detected shared gene usages for both chains, with a bias towards increased variable TRAV-12, TRAV-13, and TRBV-20 gene usages. Biased usage of TCR gene segments has been reported in the context of infection or in autoimmunity [31]. Single-cell RNA sequencing of T cells from COVID-19-infected individuals showed a preference for TRAV1-2 and TRBV20 among $CD8^+$T cells [32]. More recently, TRBV20-1 was shown to be the highest used gene segment among citrullinated peptide-specific $CD4^+$T cells from patients with rheumatoid arthritis suggesting the preference towards specific gene usages in T cell responses [33–35]. Similarly, TCR alpha chain usage (TRAV12-1) was shown to be overrepresented among $CD8^+$T cells upon yellow fever virus infection and TRAV-26-1 in celiac disease [36,37]. Our observed bias in TRAV and -BV-usages suggests a similar structural recognition mechanism.

Furthermore, the persistence of tetramer-positive $CD4^+$T cells and specific T cell clones after treatment with conventional immunosuppressive treatment suggests that the persistence of T cells with identical TCRs might explain the high risk of relapses in patients with anti-$Jo1^+$ ASyS, in support of our previous findings [13]. Future functional studies are needed to study the contribution of these antigen-specific T cells in disease progression and relapses.

One limitation of the present study is the small sample size due to the rarity of anti-$Jo1^+$ ASyS. There were also delays in patient recruitment due to the COVID-19 pandemic. Another limitation of this study was the need for *in vitro* expansion of cells before repertoire analysis, due to the rarity of autoreactive T cells. Our data reflect the part of the TCR repertoire which responded to the stimulation *in vitro*. As confirmed by our studies, there are possibly several, some yet to be discovered, immunogenic peptides/antigens on HisRS in patients with anti-$Jo1^+$ ASyS [11]. On the contrary, utilisation of HLA-class II tetramers makes it possible to identify very rare antigen-specific $CD4^+$T cells; in patients with *HLA-DRB1*03:01* with T cell reactivity against the $HisRS_{41\text{-}55}$ peptide in this case and provides a proof of concept on the detection of T cells specific for our target peptide using HLA-class II tetramers in 50% of the patients included with *HLA-DRB1*03:01* haplotype.

To the best of our knowledge, this is the first study to report the detection of autoreactive HisRS-specific T cells using peptide-HLA-class II multimer technology and paired with α/β-TCR sequencing in patients with ASyS. Identification of antigen-specific T cells has clear implications for the future development of improved therapies including tolerising and targeting pathogenic TCR signatures. Moreover, targeting shared TCR signatures might also be tested as a therapeutic approach. Finally, our tetramers could also be used as valuable tools to study the effects of treatment on presumably pathogenic antigen-specific T cells in individual patients both under conventional immunosuppressive as well as new more targeted therapies.

Competing interests

IEL has received honorarium for lecture from Janssen Pharmaceutica NV, research grant from AstraZeneca and Janssen Pharmaceutica NV, and has been serving on the advisory board for Argenx, Astra-Zeneca, EMD Serono. Research & Development Institute, Chugai, Galapagos, Novartis, Pfizer and Janssen Pharmaceutica NV and has stock shares in Roche and Novartis.

CRediT authorship contribution statement

Angeles S. Galindo-Feria: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Ravi Kumar Sharma:** Writing – review & editing, Visualization, Methodology, Data curation. **Anatoly Dubnovitsky:** Writing – review & editing, Methodology, Data curation. **Christina Gerstner:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Genadiy Kozhukh:** Methodology. **Annika Van Vollenhoven:** Writing – review & editing, Methodology. **Juan Sebastian Diaz Boada:** Writing – review & editing, Software, Methodology. **Daniel Ramsköld:** Writing – review & editing, Software. **Adnane Achour:** Writing – review & editing, Methodology. **Maryam Dastmalchi:** Resources. **Hugh H. Reid:** Writing – review & editing, Methodology. **Tatyana Sandalova:** Methodology. **Jamie Rossjohn:** Writing – review & editing, Methodology. **Karine Chemin:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Vivianne Malmström:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Ingrid E. Lundberg:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Begum Horuluoglu:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Patient consent for publication

Not applicable.

Ethics approval

The study was approved by local ethics review boards and followed the Helsinki declaration.

Provenance and peer review

Not commissioned; externally peer reviewed.

Supplementary materials

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Orcid

Angeles S. Galindo-Feria: http://orcid.org/0000-0001-7095-211X
Ravi Kumar Sharma: http://orcid.org/0000-0002-2658-0002
Anatoly Dubnovitsky: http://orcid.org/0000-0002-5350-4531
Christina Gerstner: http://orcid.org/0009-0003-4342-4241
Daniel Ramsköld: http://orcid.org/0000-0003-2892-673X
Adnane Achour: http://orcid.org/0000-0003-0432-710X
Tatyana Sandalova: http://orcid.org/0000-0002-7694-6420
Karine Chemin: http://orcid.org/0000-0002-2597-7809
Vivianne Malmström: http://orcid.org/0000-0001-9251-8082
Ingrid E. Lundberg: http://orcid.org/0000-0002-6068-9212
Begum Horuluoglu: http://orcid.org/0000-0003-2241-5170

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