Changes in biomarkers after therapeutic intervention in temporal arteries cultured in Matrigel: a new model for preclinical studies in giant-cell arteritis

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Abstract

Search for therapeutic targets in giant-cell arteritis (GCA) is hampered by the scarcity of functional systems. We developed a new model consisting of temporal artery culture in tri-dimensional matrix and assessed changes in biomarkers induced by glucocorticoid treatment.

Reference


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EXTENDED REPORT

Changes in biomarkers after therapeutic intervention in temporal arteries cultured in Matrigel: a new model for preclinical studies in giant-cell arteritis

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ABSTRACT

Background Search for therapeutic targets in giant-cell arteritis (GCA) is hampered by the scarcity of functional systems. We developed a new model consisting of temporal artery culture in tri-dimensional matrix and assessed changes in biomarkers induced by glucocorticoid treatment.

Methods Temporal artery sections from 28 patients with GCA and 22 controls were cultured in Matrigel for 5 days in the presence or the absence of dexamethasone. Tissue mRNA concentrations of pro-inflammatory mediators and vascular remodelling molecules was assessed by real-time RT-PCR. Soluble mediators were measured in the supernatant fluid by immunoassay.

Results Histopathological features were unequivocally preserved in cultured arteries. mRNA concentrations of pro-inflammatory cytokines (particularly IL-1β and IFNγ), chemokines (CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) and MMP-9 as well as IL-1β and MMP-9 protein concentrations in the supernatants were significantly higher in cultured arteries from patients compared with control arteries. The culture system itself upregulated expression of cytokines and vascular remodelling factors in control arteries. This minimised differences between patients and controls but underlines the relevance of changes observed. Dexamethasone downregulated pro-inflammatory mediator (IL-1β, IL-6, TNFα, IFNγ, MMP-9, TIMP-1, CCL3 and CXCL8) mRNAs but did not modify expression of vascular remodelling factors (platelet derived growth factor, MMP-2 and collagens I and III).

Conclusions Differences in gene expression in temporal arteries from patients and controls are preserved during temporal artery culture in tri-dimensional matrix. Changes in biomarkers elicited by glucocorticoid treatment satisfactorily parallel results obtained in vivo. This may be a suitable model to explore pathogenetic pathways and to perform preclinical studies with new therapeutic agents.

INTRODUCTION

Giant-cell arteritis (GCA) is a granulomatous arteritis of the elderly, targeting the aorta and its branches with a striking tropism for the cranial arteries.1 Although most patients with GCA experience a remarkable relief with high-dose glucocorticoids (GC), treatment has proven to be unsatisfactory. GC fail to prevent further sight deterioration in 10%–17% of patients presenting with visual impairment and are unable to avoid large vessel damage leading to aortic dilatation in about 22.5% of patients.2,3 Moreover, more than 50% of patients relapse when GC are tapered4–5 and GC-related adverse events occur in a more than 80% of patients with GCA.6 There is an unmet need for more effective and specific therapies.

Search for therapeutic innovation in GCA is difficult due to the limited understanding of pathogenesis and the scarcity of functional models where the impact of therapeutic interventions can be assessed. The pathogenesis model of GCA is based on the identification of particular cell types (CD4 T lymphocytes, macrophages, dendritic cells, endothelial cells),7,8 cell activation and differentiation markers,7,9 and inflammatory mediators in lesions.9–13 The interpretation of immunopathology findings is often extrapolated from basic immunology principles, and the role of infiltrating cells and their products is assumed from their known biological activities and association with particular phenotypes,10–11 histopathological changes or outcomes.12–14 Proof of concept is weak for the majority of grounds on which the current pathogenetic model is sustained.

The frustrating experience with anti-tumour necrosis factor (TNF) therapy in GCA underlines the crucial need for functional systems. TNFα was considered a potential therapeutic target based on its strong upregulation in lesions13 and correlation of tissue and serum TNFα levels with GC requirements and relapsing course.13,15 In spite of these observations and in spite of the therapeutic efficacy of TNF blockers in other granulomatous diseases, neutralising TNFα with infliximab did not seem sufficient to abrogate inflammatory activity in GCA.5 Blocking IL-6 receptor is currently being considered as a therapeutic option.16 This and other interventions could benefit from preclinical functional testing.

A functional model was created by Brack et al17 subcutaneously engrafting fragments of human temporal arteries into severe combined immunodeficiency (SCID) mice. This pioneer model has been useful to detect changes in cytokine expression in temporal artery tissue after pharmacological...
treatment of engrafted mice\textsuperscript{18 19} or after selective depletion of specific cell types with antibodies injected to animals,\textsuperscript{8 17} providing proof of concept of some of the basic pathogenic principles. However, this model is complex, expensive and not widely available. Moreover, monitoring of successful engraftment is difficult and the accessibility of therapeutic agents administered to the mice cannot be controlled. Due to its complexity, the majority of published experiments have been performed with only 1–3 temporal arteries split into several mice.\textsuperscript{8 18 19}

The Engelbreth-Holm-Swarm sarcoma-derived tri-dimensional matrix, Matrigel\textsuperscript{20} provides anchorage and survival signals for vascular smooth muscle cells (VSMC).\textsuperscript{21} Based on these findings, we developed a new model to assess changes in lesions after therapeutic intervention, consisting of culture of temporal artery sections embedded in Matrigel. We found that cultured arteries remained viable for at least 2 weeks with excellently preserved morphology. Moreover, this system was sensitive enough to demonstrate clear differences in cytokine expression between normal and inflamed arteries as well as changes induced by therapeutic intervention.

METHODS

Patients

Temporal artery biopsies were performed to 50 consecutive patients with suspected GCA for diagnostic purposes. A 5–13 mm segment was saved for this study and the remaining fragment was processed for histopathological diagnosis. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona and patients signed informed consent.

A total of 28 biopsies disclosed histopathological features of GCA and 22 revealed no inflammatory infiltrates. Patients with a negative temporal artery biopsy were eventually diagnosed with other conditions (see online supplementary methods).

Temporal artery culture

Temporal artery fragments were placed in RPMI 1640 medium (Lonza; Verviers, Belgium) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, California, USA), 2 mM L-glutamine (Invitrogen), amphotericin B at 2.5 μg/ml (Invitrogen) and gentamycin (Braun, Germany) at 200 μg/ml. An average of 10.79±2.91 (mean±SEM) 0.8 μl Matrigel drop per well, which was allowed to solidify at 37°C for 30 min. One temporal artery section per well was placed in Matrigel and covered with 1 ml medium. Dexamethasone (Sigma, Ayrshire, UK) at 0.5 μg/ml was added to selected wells. Each condition was incubated at 37°C in 5% CO₂ for 5 days. Replicates of supernatant fluids and biopsies were respectively pooled. Biopsies were frozen in TRIzol reagent (Invitrogen) and stored at −80°C. Random specimens were cultured for 2 weeks in order to assess morphology preservation after extended culture periods and fixed in 10% formalin for H&E staining and histopathological examination.

Immunostaining

Cultured temporal artery sections were de-paraffinised, washed in phosphate-buffered saline (PBS) and endogenous peroxidase was blocked with H₂O₂. Slides were incubated with mouse antihuman CD3 (clone PS1, Leica Microsystems, Wetzlar, Germany, 1:60 dilution) or undiluted mouse antihuman CD68 (clone KP1 from Dako, Glostrup, Denmark, ready to use). Optimal dilutions were tested on human tonsils (positive control). Isotype-matched mouse immunoglobulins served as negative controls. Immunodetection was performed with a HRP-labelled polymer conjugated to a secondary antibody (EnVision, Dako) using 3`-3`-diaminobenzidine as a chromogen.

Cytokine mRNA measurement by real-time quantitative RT-PCR

Three to four temporal artery sections per condition were homogenised in TRIzol reagent. RNA extraction was performed according to the chloroform-isopropanol precipitation method. Total RNA (1 μg) was reverse transcribed to cDNA using Archive kit (Applied Biosystems, Life Technologies, Carlsbad, California, USA) in a final volume of 100 μl, employing random hexamer priming. Samples were stored at −80°C until use.

Gene expression of pro-inflammatory cytokines (IL-1β, IL-6, TNFα, interferon (IFN)γ), chemokines (chemokine ligand (CCL)2/monocyte chemoattractant protein (MCP)-1, CCL3/ MCP-1α, CCL4/MIP1β, CCL5/regulated upon activation normal T cell expressed and secreted (RANTES) and CXCL8/IL-8), metalloproteases (matrix metalloproteinases (MMP)-2, MMP-9) and their inhibitors (tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2), growth factors (platelet derived growth factor (PDGF) A and B) and vascular matrix components (collagen I, collagen III) was assessed using specific predeveloped Taqman probes from Applied Biosystems (Taqman Gene Expression Assays; see online supplementary methods). Fluorescence was detected with ABI PRISM 7900 Sequence Detection system and results were analysed with the Sequence Detection Software V2.3 (Applied Biosystems). Comparative Ct method was used to assess the relative gene expression. All samples were normalised to the expression of the endogenous control GUSb and values were expressed as relative units.

Detection of inflammatory mediators in the supernatant fluid by immunoassay

Pro-inflammatory cytokines (IL-6, TNFα, IL-1β, IFN)γ, chemokines (CCL2/MCP-1 and CCL3/MIP-1α), metalloproteases (MMP2 and MMP-9) and growth factors (PDGF AB) were detected by enzyme-linked immunoassay (Quantikine ELISA kits from R&D Systems, Minneapolis, Minnesota, USA) in the supernatant fluids from 10 patients and six controls.

Statistical analysis

Mann–Whitney test was applied for statistical analysis.

RESULTS

Viability of the system and preserved morphology

Arterial sections were daily monitored under an inverted microscope. In GCA arteries white blood cells were visible in the periphery of the artery and remained bright and viable as assessed by Trypan blue exclusion throughout the duration of the experiment (figure 1A). After 1 week, VSMC began to spread and extend towards the matrix, further supporting the viability of this system (figure 1B).

As shown in figure 1C and 1D histopathological examination of cultured GCA arteries disclosed that morphological details including distinct arterial layers, inflammatory infiltrates, internal elastic lamina fragments and giant-cells were perfectly preserved. Over time, the intensity of inflammatory infiltrates decreased in cultured arteries, as described in arteries engrafted into SCID mice.\textsuperscript{17} As shown in figure 1E–H, inflammatory...
infiltrates, including giant-cells, decreased after 2-week culture. Examination of the cultured arteries under an inverted microscope disclosed that, over time, some inflammatory cells migrated along the outgrowing VSMC (figure 1B).

Differences in expression and release of relevant molecules between cultured GCA and control arteries

To assess the model reliability we investigated expression of pro-inflammatory cytokines, chemokines, metalloproteinases and...
growth factors largely known to be expressed in GCA lesions and thought to be relevant to pathogenesis. We also explored some additional chemokines, such as CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CXCL8/IL-8, not previously investigated in GCA.

After a 5-day incubation period, remarkable differences in the spontaneous expression and release of various relevant factors were detected between GCA and control arteries, underlining the accurate sensitivity of the system to distinguish between non-inflamed and inflamed arteries (table 1). Differences in gene expression were particularly significant for IL-1β, IFNγ, chemokines CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES, and MMP-9. Less marked or no differences were observed for other factors known to be upregulated in GCA lesions including IL-6, TNFα and CCL2. Intense expression by cultured control arteries probably minimised differences.

Of interest, MMP-9, mainly produced by inflammatory cells, was overexpressed in patient versus control specimens whereas MMP-2, constitutively expressed by VSMC, was similar between patients and controls, paralleling again what has been observed in uncultured temporal artery biopsies.22 As previously observed in freshly removed arteries, TIMP-1 and TIMP-2 mRNAs were decreased in inflamed arteries, leading to increased proteolytic balance.23 Vascular remodelling factors PDGFs, CCL2, MMP-2 and collagen I and III were markedly expressed in control arteries whereas IFNγ and collagens decreased in GCA specimens (figure 2). In general, the culture system minimised differences between patients and controls.

Variations in the secretion of various markers were observed. TNFα and particularly IL-6 were remarkably released in the supernatant fluid (table 1). However, IFNγ and IL-1β, markedly expressed at the mRNA level, were secreted in small amounts. This parallels what happens in vivo where circulating TNFα and IL-6 are increased in sera of patients whereas IL-1β and IFNγ are not easily secreted and remain around the detection threshold in human serum. Therefore, this system allows evaluation of cytokine expression and investigation of cytokine secretion.

Similarly, while there were significant differences in chemokines CCL3/MIP1-α, CCL4/MIP-1β and CCL5/RANTES between patients and controls at the mRNA level, differences in released chemokines were less apparent.

### Effect of the culture system on gene expression in cultured arteries

Since control arteries notably expressed various mediators we next investigated whether the culture system itself influenced gene expression. Frozen tissue from the original artery was available for six of the GCA patients and five controls and the expression of selected markers was compared between sections of the same specimen before and after 5-day culture in Matrigel. With the exception of IFNγ, the culture system upregulated expression of pro-inflammatory cytokines, chemokines CCL2 and CXCL8, and MMP-9 in both patients and controls. PDGFs and collagen III were markedly increased in control arteries whereas IFNγ and collagens decreased in GCA specimens (figure 2). In general, the culture system minimised differences between patients and controls.

### Effect of dexamethasone on inflammatory infiltrates and on the expression and release of inflammatory and vascular remodelling markers

To assess whether this ex vivo system allowed accurate detection of changes induced by pharmacological intervention, we compared expression and release of inflammatory markers between artery sections cultured with medium alone and sections from 10 patients cultured in the presence of dexamethasone. A marked decrease in cytokine production was observed (figure 3 and table 2). Chemokines were downregulated at the mRNA level but changes in chemokine release were, again, less apparent (table 2). Vascular remodelling factors such as CCL2, MMP-2 and PDGF as well as collagens I and III were not downregulated by dexamethasone.

Dexamethasone treatment for 2 weeks induced a decrease in macrophage infiltration as assessed by CD68 mRNA expression and immunohistochemistry (figure 4). No effect on T cells was observed during the same treatment period.

### Table 1 Differences in biomarker mRNA expression (relative units) and protein secretion (pg/ml) between cultured temporal artery sections from GCA patients and controls

<table>
<thead>
<tr>
<th>mRNA concentration (relative units)</th>
<th>Protein concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GCA biopsies</strong></td>
<td><strong>Control biopsies</strong></td>
</tr>
<tr>
<td>IL-1β</td>
<td>35.9±8.80</td>
</tr>
<tr>
<td>IL-6</td>
<td>448.5±86.88</td>
</tr>
<tr>
<td>TNFα</td>
<td>4.7±2.26</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.8±0.25</td>
</tr>
<tr>
<td>CCL-2/MCP-1</td>
<td>648.7±155.2</td>
</tr>
<tr>
<td>CXCL8/IL-8</td>
<td>228.7±619.9</td>
</tr>
<tr>
<td>CCL-3/MIP-1α</td>
<td>86.3±16.9</td>
</tr>
<tr>
<td>CCL-4/MIP-1β</td>
<td>28.2±6.13</td>
</tr>
<tr>
<td>CCL-5/RANTES</td>
<td>139.8±37.4</td>
</tr>
<tr>
<td>MMP-2</td>
<td>2097.4±276.9</td>
</tr>
<tr>
<td>MMP-9</td>
<td>1283.8±408.2</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>11813±3550</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>586.6±87.7</td>
</tr>
<tr>
<td>COL I</td>
<td>1554.6±284.6</td>
</tr>
<tr>
<td>COL III</td>
<td>3674.4±370.7</td>
</tr>
<tr>
<td>PDGF A</td>
<td>71.1±4.7</td>
</tr>
<tr>
<td>PDGF B</td>
<td>40.7±8.7</td>
</tr>
<tr>
<td>PDGF AB</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

Values in bold are statistically significant (p<0.05). mRNA expression was detected in the entire cohort of 28 GCA patients and 22 controls. CCL3/MIP-1α, CCL4/MIP-1β and CXCL8/IL-8 protein concentrations were detected by Luminex in 10 patients and six controls. The remaining proteins were detected by ELISA in the entire cohort. COL, Collagen; GCA, giant-cell arteritis.
DISCUSSION

Functional models are essential to explore pathogenic pathways and to test therapeutic intervention in diseases. We developed a new model of temporal artery culture in tri-dimensional matrix to perform functional studies in GCA. Short-term explant culture of involved tissue has been previously used in other conditions such as rheumatoid arthritis and has provided useful insights into involved immunopathogenic pathways. A previous attempt of culturing temporal artery explants was tried by Blain et al. However, without the use of a supporting matrix, the specimen remained viable for a short period of time. Specimens were cultured for 20 h only and the release of media-fuids had to be induced with lipopolysaccharide which is an important exogenous manipulation.

The main innovation of our culture system is the embedding of the specimen in Matrigel which supports viability with active production of inflammatory mediators and their spontaneous release into the culture medium. In addition to provide an anchorage system for the wounded VSMC medial layer of the excised sections, Matrigel provides survival and proliferation signals for VSMC which, in turn, may promote survival of infiltrating lymphocytes and macrophages. In this model, morphology was excellently preserved within 2-week culture.

There was a remarkable variability in the spontaneous production of inflammatory mediators, reflecting the notable differences in the density of inflammatory infiltrates and individual variation in cytokine production existing among patients with GCA. This observation underlines the need of a suitable model where testing specimens from multiple donors is feasible.

Spontaneous expression of IL-1β, IFNγ, MMP-9 and chemokines CCL3, CCL4 and CCL5 was significantly higher in explants from patients compared with controls and closely paralleled what has been described in immunopathology studies of freshly removed GCA arteries.

GC substantially reduced the production of pro-inflammatory cytokines IL-1β, IL-6 and TNFα both at the mRNA and protein level and also IFNγ mRNA. Expression of chemokines was also markedly decreased. These changes were similar to what has been observed in cross-sectional comparisons in biomarker expression between biopsies obtained from untreated patients and biopsies from patients who have already received GC, or results obtained in temporal artery biopsies engrafted in the SCID mice. GC treatment induced also a decrease in macrophage infiltration, whereas virtually no effect was observed on T cells, suggesting that T cell infiltration may be more resistant to GC therapy.

An interesting contribution of this study is that the expression of vascular remodelling factors such as CCL2/MCP-1, MMP-2, PDGFs and collagen I and III is not influenced by GC. A previous study comparing sequential biopsies obtained in four patients before and after 1 year of GC treatment showed, indeed, that vascular remodelling factors increased after long-term GC treatment. This may explain why some patients
continue to develop vascular occlusive events in spite of GC treatment.2

A limitation of this model is that Matrigel itself, by promoting survival and proliferation of smooth muscle cells, may directly influence the expression or detection of some products introducing a bias in the results. The culture system downregulated IFNγ and collagen I expression in GCA arteries and, conversely, upregulated the expression IL-6, CCL2/MCP-1, MMP-9, CXCL8/IL-8, PDGFs and collagen III in control arteries. These molecules may be part of vascular remodelling/repair programme stimulated by surgical injury and facilitated by attachment to the matrix. These observations indicate that some differences in gene expression observed between patients and controls are minimised by the culture system but, at the same time, enhance the significance of the differences observed.

Furthermore, this finding underlines the need of investigating how the culture system influences the expression of any factor to be tested in this model.

Another limitation is that detection of some mediators such as chemokines in the culture medium may not accurately reflect their actual production. Chemokines act in an autocrine/paracrine manner and interact with matrix proteins to create a local

### Table 2

Changes in biomarker protein concentration (pg/ml) in the supernatant fluid from untreated cultured GCA temporal artery sections and cultured GCA sections exposed to dexamethasone

<table>
<thead>
<tr>
<th>Protein concentration (pg/ml)</th>
<th>Untreated artery sections (mean ±SEM)</th>
<th>Dexamethasone-treated artery sections (mean ±SEM)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>6.06±1.32</td>
<td>2.41±0.76</td>
<td>0.017</td>
</tr>
<tr>
<td>IL-6</td>
<td>31059.8±10600.6</td>
<td>4796.5±1968.4</td>
<td>0.020</td>
</tr>
<tr>
<td>TNFα</td>
<td>27.043±6.398</td>
<td>2.22±2.104</td>
<td>0.041</td>
</tr>
<tr>
<td>IFNγ</td>
<td>4.901±1.8</td>
<td>4.792±1.66</td>
<td>0.961</td>
</tr>
<tr>
<td>CCL2/MCP-1</td>
<td>11759±2679.5</td>
<td>5130.2±598.83</td>
<td>0.921</td>
</tr>
<tr>
<td>CCL3/MIP-1</td>
<td>24.11±9.05</td>
<td>19.41±11.38</td>
<td>0.399</td>
</tr>
<tr>
<td>CXCL8/IL-8</td>
<td>132670.4±41358.1</td>
<td>2465.5±631.76</td>
<td>0.056</td>
</tr>
<tr>
<td>MMP-2</td>
<td>39.125±11.14</td>
<td>27.5±7.79</td>
<td>0.204</td>
</tr>
<tr>
<td>MMP-9</td>
<td>48.91±10.74</td>
<td>8.64±1.89</td>
<td>0.003</td>
</tr>
<tr>
<td>PDGF AB</td>
<td>23.38±3.25</td>
<td>20±2.83</td>
<td>0.394</td>
</tr>
</tbody>
</table>

GCA, giant-cell arteritis.

Figure 3 Changes in biomarker mRNAs induced by dexamethasone treatment. Comparison in mRNA concentration of selected biomarkers between untreated temporal artery sections from the giant-cell arteritis cohort (white box) and temporal artery sections from 10 of the patients subjected to dexamethasone at 0.5 mg/ml (grey box). *p<0.05; **p<0.005.
Figure 4  Effects of dexamethasone treatment on the density of infiltrating T lymphocytes and macrophages. (A) Differences in mRNA concentration of CD3 (T lymphocyte marker) and CD68 (macrophage marker) between 28 untreated giant-cell arteritis (GCA) temporal artery sections and 10 GCA sections exposed to 0.5 μg/ml dexamethasone. *p=0.059. (B) Changes in infiltrating T lymphocytes (identified by anti-CD3 immunostaining) and macrophages (identified by anti-CD68 immunostaining) upon dexamethasone treatment. (C) CD3 or CD68 cell number per field in three paired arteries cultured with or without dexamethasone. **p=0.004.
gradient. Therefore, chemokines may be retained in the artery and surrounding proteoglycan-rich matrix, according to their physiological function.\textsuperscript{25} Dissociation between tissue and serum concentrations of relevant chemokines has been observed in several chronic inflammatory conditions.\textsuperscript{12}

Our model overcomes some of the limitations of the temporal artery engraftment into the SCID mice. It allows daily monitoring of viability, it ensures direct accessibility of the molecules tested, it allows serial detection of proteins secreted into the culture medium and morphology is better preserved. Since retrieval of the cultured specimens is direct and simple, very thin sections can be used, allowing the assessment of replicates to assure consistency, and the testing of various conditions per specimen. This is very important given the remarkable variability in the intensity of inflammatory infiltrates and cytokine production among patients. In addition, it is cheap, easy, spares animals and does not require special equipment besides tissue culture facilities. In fact, since the initial communication of preliminary results,\textsuperscript{26, 27} this model is being used by other investigators.\textsuperscript{28} It shares with the SCID mice model the limitation that only changes in biomarkers can be assessed and true, clinically relevant, disease outcomes cannot be investigated.

In summary, we developed an artery explant culture system based on the unique properties of Matrigel in creating a three-dimensional matrix support and promoting VSMC survival. This method is sensitive enough to detect changes after intervention and may be useful to explore pathogenic pathways and to assess the impact of new therapeutic agents.

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Contributors
MCC and MUR designed the study. J-MD contributed important input to its design. MC-B, AG-M, EL, EP-R and PR-L performed the experimental work. GE-F, SP-G, MAA, JH-R and MB contributed to clinical selection and contributed to the experimental work. PLF supervised the immunopathology studies. All authors evaluated and criticised the data and J-MD provided important contributions to their interpretation. MC-B and MCC wrote the manuscript. All authors read, made improvements and approved the final version.

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Competing interests
MUR is a full employee by Pfizer Inc. No other competing interests.

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