therefore was attributed solely to the spectroscopic interference present.

At MB concentration of 0.5 mg/L, mimicking the typical therapeutic MB concentration 30 min after antidote administration, no significant interference was observed. Given the onset time for MB's action of 30 min,[10] it could be safely concluded that the interference on MetHb measurement would be minimal if blood taking is arranged 30 min after giving the MB antidote. In clinical practice, after MB treatment, patients with methaemoglobinaemia are monitored for resolution of symptoms and with serial MetHb measurements.[11] Repeated blood testing for MetHb is usually made in 1−3 h.[10,12] The results from our study are therefore in-line with the current practice, and could help reassure clinicians on the accuracy of MetHb concentration measured.

At MB concentration of 5 mg/L, which simulated the maximal MB concentration at 5 min after antidote administration, MetHb concentrations at >20% (levels 2, 3 and 4) were relatively unaffected. In contrast, a significant negative interference (absolute difference of −4.0% unit, equivalent to 27.2% relative decrease from the control aliquot level) was observed when MetHb concentration was <20% unit (level 1). Therefore, clinicians should be advised not to recheck MetHb concentration immediately after MB administration, as this may misguide clinical judgement and any subsequent management plan.

At MB concentration of 20 mg/L, a significant negative interference (absolute difference of −14.2% unit, equivalent to 42.4% relative decrease from the control aliquot level) was observed at lower MetHb concentration at approximately 30% unit (levels 2). At level 1 with MetHb concentration of approximately 10%, MB spiking even introduced an error signal and the MetHb level in this spiked sample could not be determined. Higher MetHb concentrations were relatively free from MB interference (levels 3 and 4). These data were consistent with the findings by Gourlain *et al.*[2] and Dötsch *et al.*[3] Modern co-oximetry, despite the improved technical specifications, is still subjected to MB interference at a very high concentration. However, this phenomenon is unlikely clinically significant as such MB concentration is much higher than the usual therapeutic range.

Several limitations existed in the current study. First, our interference study was carried out on the ABL800 FLEX co-oximetry only. It may not be possible to generalise our findings to other analysers. For laboratories using other models of co-oximetry it may be worth performing independent studies to evaluate the susceptibility of their analysers to MB interference. Second, the MetHb concentrations in the blood samples were not equally spaced, and they differed slightly between spiking set-ups. This could be attributed to the minor variations in incubation time and temperature. Third, in order to perform simultaneous MetHb measurement on both the spiked and its corresponding control samples, two independent blood gas analysers were employed, which may have introduced a larger CV_A.

In conclusion, an interference study of varyingly spiked MB concentrations on measurement of MetHb at 10−60% unit using ABL800 FLEX was performed. No interference was observed on MetHb measurement at the usual therapeutic concentration of 0.5 mg/L MB. A significant negative interference could be observed on MetHb measurement when MB was spiked at 5 mg/L, simulating the peak concentration after an intravenous dose. Clinicians are advised not to recheck MetHb concentration immediately after MB administration. However, they can be reassured that the measured MetHb concentration is not subjected to any significant MB interference 30 min after intravenous MB administration.

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A lesson learnt from laboratory error: isoelectric focusing improves the accuracy in interpreting polyclonal or monoclonal IgG4

To the Editor,
IgG4-related disease (IgG4-RD) is an immune-mediated condition characterised by fibroinflammatory lesions and

elevated polyclonal IgG4. It often presents as a multiorgan disease with myriad clinical presentations, and mimics many malignant, infectious, or other immune-mediated disorders.[1] The diagnosis of IgG4-RD mainly relies on the histopathological analysis of tissue biopsy, including the three central pathology features of lymphoplasmacytic infiltration enriched with IgG4-positive plasma cells (IgG4/IgG ratio >40%), storiform fibrosis, and obliterative phlebitis.[2]

Since IgG4-RD was first recognised as a distinct disease in 2003, it has emerged as an important cause of polyclonal hypergammaglobulinaemia. Elevated serum concentrations of IgG4 are useful in diagnosing IgG4-RD but are neither sufficiently sensitive nor specific. High polyclonal IgG4 levels are found in 60−70% of patients with IgG4-RD; however, about 20% of patients with IgG4-related autoimmune pancreatitis have normal serum IgG4 concentrations at presentation.[3] Mildly elevated serum IgG4 is non-specific, but markedly elevated serum IgG4 (>5 g/L) is highly suggestive of IgG4-RD and warrants further investigation, including histological confirmation in most cases. Serum protein electrophoresis (SPE), immunofixation electrophoresis (IFE), and serum free light chains (sFLCs) tests are very helpful tests to differentiate polyclonal hypergammaglobulinaemia from monoclonal gammopathy.[4,5] On occasion, it can be difficult to distinguish monoclonal IgG4, as in IgG4 multiple myeloma (MM) or monoclonal gammopathy of undetermined significance (MGUS), from the polyclonal hyper-IgG4 in IgG4-RD. Herein, we present the use of isoelectric focusing (IEF) to aid in determining if the observed electrophoretic migration pattern of IgG4 is derived from either polyclonal or monoclonal IgG.

A 70-year-old male presented with disseminated varicella zoster virus (VZV) infection, weight loss and fatigue in August 2020. While living in Nepal in 2017, he had been diagnosed with sarcoidosis based on bilateral parotid and submandibular gland swelling and mediastinal lymphadenopathy on CXR. A fine needle aspirate of his salivary gland had been reported as 'chronic sclerosing sialadenitis' but no sarcoid granuloma was reported. He had been treated with 6 months of prednisolone and methotrexate, then continued on hydroxychloroquine. His VZV was treated with valaciclovir with rapid improvement. However, his parotidomegaly and history of mediastinal lymphadenopathy raised the possibility of IgG4-RD, so he underwent further investigations.

Serum immunoglobulin analysis revealed markedly elevations of IgG and IgG4 at 40.70 g/L [reference interval (RI) 7.00−16.00] and 29.10 g/L (RI 0.03−2.01), respectively. sFLC studies showed increased concentrations for both kappa and lambda light chains while the kappa/lambda ratio was normal. Results of other autoimmune screening including antinuclear antibodies (ANA), antineutrophil cytoplasmic antibodies (ANCA) and anti-extractable nuclear antigen antibodies (ENA), were negative. Biclonal IgG kappa and lambda paraproteins (concentration of 21.2 g/L) were initially reported based on the patterns observed on agarose SPE (Fig. 1A,B: Lane 2) and IFE (Fig. 1C) performed using the Interlab G26 automated instrument (Interlab, Italy).

His positron emission tomography (PET) showed fluorodeoxyglucose (FDG) avidity of parotids, mediastinal and hilar nodes, and around the lower aorta and common iliac vessels (Fig. 2A). Core biopsies of his parotids revealed an extensive chronic inflammatory infiltrate with lymphocytes, plasma cells and bands of sclerosis (Fig. 2B). IgG and IgG4 staining revealed a high proportion of IgG4-positive plasma cells (Fig. 2C,D), although inconclusive IgG4/IgG ratio due to the density of the infiltrate.

With a profound, disproportionate elevation of serum IgG4 of 29.1 g/L (>10 times upper limit of normal), clinical manifestations, and histological and radiological findings, the patient was highly suspicious of IgG4-RD despite the initial 'sarcoidosis' clinical diagnosis and reported biclonal paraprotein serological result. Based on the 2019 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) IgG4-RD classification criteria,[6] the patient scored 35 inclusion points, compared to the cut-off criteria of 20 points. Therefore, we considered the diagnosis of IgG4-RD highly likely.

However, the initial laboratory report of a biclonal gammopathy was not consistent with the clinical suspicion of IgG4-RD. Reviewing the SPE and IFE gels, we considered there were multiple reasons for the misinterpretation of the patterns as a biclonal gammopathy. Firstly, laboratory staff were unfamiliar with the specific IgG4 migration pattern on agarose SPE. Thus, the relatively focal but restricted band in bridging of beta and gamma fractions in SPE (Fig.1A: Lane 2) was mistakenly considered as monoclonal band. Secondly, patient IFE demonstrated the focal band consisted of IgG with suppression of IgA and M. Both kappa and lambda light chains demonstrated similar focal restriction and were interpreted as monoclonal bands. Thirdly, increased concentrations for both kappa and lambda light chains with a normal ratio led to an incorrect consideration of biclonal production.

Human immunoglobulins and four IgG subclasses are known to have distinctive electrophoretic migration patterns in SPE.[7] Elevated serum IgG4, polyclonal or monoclonal, always migrate as a focal band at the anodal end of the gamma fraction in SPE. A sharp, discrete monoclonal band is very helpful in differentiating IgG4 MM or MGUS from IgG4-RD. However, this is not always the case in IgG4 MGUS or MM in remission or with a low tumour burden; SPE can demonstrate focal but somewhat broad, beta-gamma bridging similar to that of IgG4-RD. On the other hand, a focal band caused by markedly elevated polyclonal IgG4 seen in IgG4-RD may be misinterpreted as a monoclonal or biclonal gammopathy.[8−10] Moreover, skewing of the polyclonal IgG4 antibodies toward either kappa or lambda can further imitate a monoclonal appearance on IF analysis.[8] Thus, it is critical to differentiate monoclonal gammopathy from polyclonal hypergammaglobulinaemia in patients with elevated serum IgG4, as incorrect classification could lead to unnecessary investigations, delayed diagnosis and incorrect treatment.

Agarose isoelectric focusing (IEF) is far superior to SPE for the detection of monoclonal or oligoclonal immunoglobulins even when present at low concentrations of <0.05 g/L or masked by polyclonal immunoglobulin. IEF is widely used in the analysis of CSF oligoclonal IgG bands but can also be applied to the detection of monoclonal IgG in serum. The broad range pH gradient separates proteins according to their different isoelectric points, with the concentration of oligoclonal and monoclonal immunoglobulin into a limited number

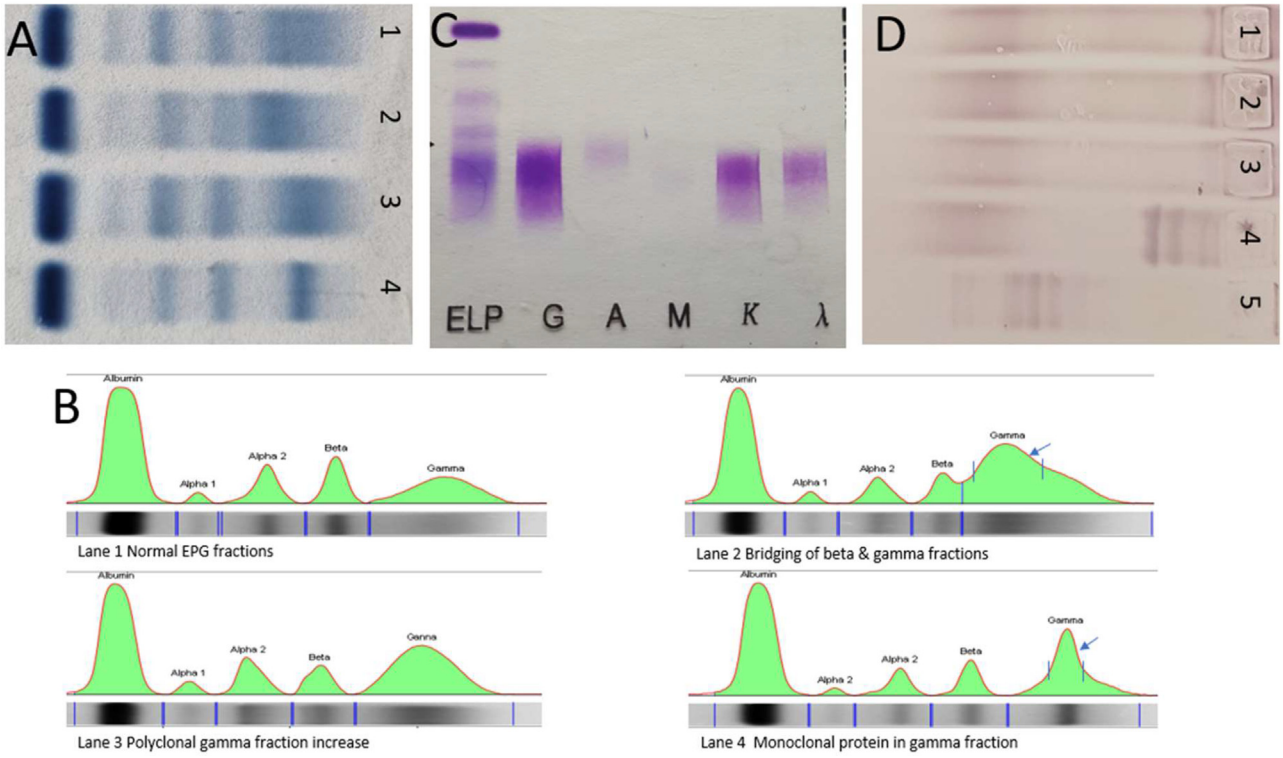

Fig. 1 Serum protein electrophoresis (SPE), immunofixation electrophoresis (IFE) and isoelectric focusing (IEF) findings. (A) SPE. Lane 1, normal control serum. Lane 2, patient serum. Lane 3, polyclonal hypergammaglobulinaemia from a patient with liver disease. Lane 4, monoclonal IgG kappa from a patient with multiple myeloma. (B) Corresponding electropherograms of (A). Lane 2, arrow indicates the characteristic relatively focal but restricted mobility of IgG4 in bridging of the beta and gamma fractions. Lane 4, arrow indicates a characteristic sharp band of 10 g/L monoclonal IgG kappa. (C) Patient IFE with fixative (SPE-ELP lane), anti-human IgG, IgA, IgM, kappa and lambda, respectively. (D) IEF and immunoblotting. Lane 1, normal polyclonal IgG serum, diluted to an IgG concentration of 0.04 g/L. Lanes 2 and 3, patient sera with final IgG concentrations of 0.06 g/L and 0.03 g/L, respectively. Lane 4, monoclonal IgG kappa serum diluted to an IgG concentration of 0.04 g/L. Lane 5, positive oligoclonal IgG control.

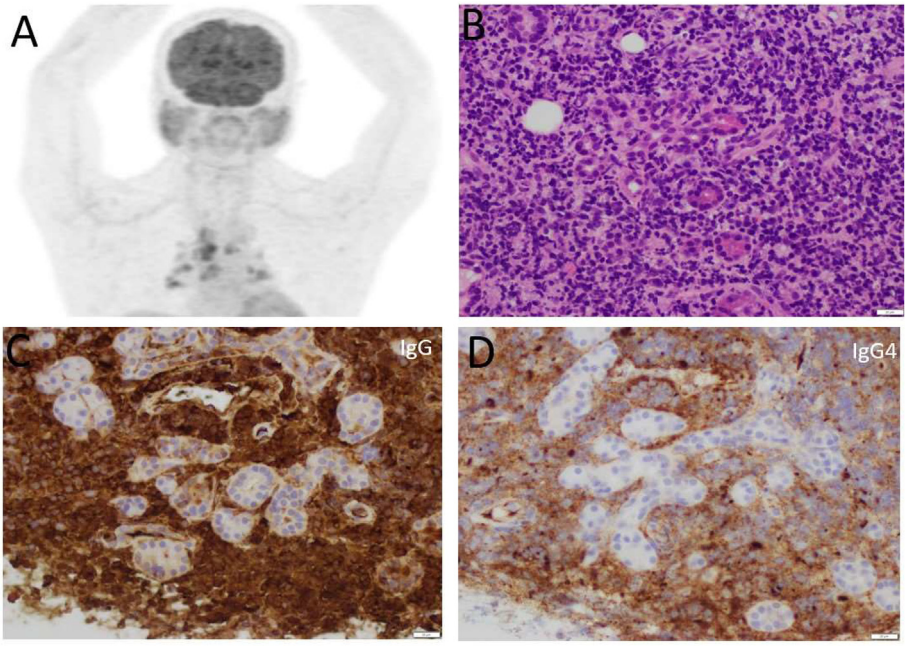

Fig. 2 Imaging and right parotoid histopathology findings. (A) FGD-PET image of patient. (B) Haematoxylin and eosin stain. (C,D) IgG and IgG4 immunostain.

of small tight bands and spreading of polyclonal immunoglobulin. Monoclonal IgG produces a very specific ladder pattern of four to five bands equally spaced from each other on IEF, with descending intensity toward the anodic region of the blot.[11] The patient's IEF and immunoblot results demonstrated the typical polyclonal IgG spreading pattern (Fig. 1D: Lane 2,3) that could be easily distinguished from the typical ladder pattern of monoclonal IgG (Fig. 1D: Lane 4) and oligoclonal pattern (Fig. 1D: Lane 5). Subsequently, the SPE and IFE results were amended to polyclonal increase in gamma fraction, which was concordant with the clinical impression of IgG4-RD.

Although the interpretation of elevated IgG4 by SPE and IFE can be challenging in some sera, there are a few key points that should be considered. Firstly, beta-gamma bridging on SPE in this patient was strongly skewed toward the anodal region of the gamma fraction, which is consistent with the electrophoretic migration of IgG4. This can be clearly distinguished from the typical hypergammaglobulinaemia associated with infectious or inflammatory processes (Fig. 1A: Lane 3). It can also be distinguished from the beta-gamma bridging found in liver disease, which is associated with a polyclonal increase in IgA. Secondly, IEF is a useful technique to assist in identifying a focal band as polyclonal or monoclonal, when doubt exists after performance of SPE and IFE. Lastly, the sFLC kappa/lambda ratio plays an important role in distinguishing monoclonal from polyclonal hypergammaglobulinaemia. In patients with polyclonal hypergammaglobulinaemia or renal impairment, free kappa and lambda concentrations may increase due to increased immune production or decreased clearance. This results in an increase in both kappa and lambda equally, thereby maintaining a constant ratio. In contrast, only one of the FLC concentrations increases in monoclonal gammopathy, typically demonstrating a significant abnormal ratio. However, studies have shown that FLC ratio can be strongly abnormal in patients with IgG4-RD.[8,12] Thus, sFLC analysis may be less helpful than expected in differentiating polyclonal or monoclonal IgG4.

This patient's case was instructive on two levels: first the clinical misdiagnosis of sarcoidosis instead of IgG4-RD, then a laboratory misinterpretation of IgG4 biclonal gammopathy instead of a polyclonal IgG4 hypergammaglobulinaemia. We share this case to alert colleagues to the potential for misdiagnosis.

The accurate interpretation of polyclonal IgG4 is important for the diagnosis of IgG4-RD. Greater awareness of the difference in IgG subclass electrophoretic migration patterns in SPE and IFE is beneficial for the laboratory. In patients with markedly elevated polyclonal IgG4 (>5 g/L), a characteristic relatively focal, but restricted beta-gamma bridging pattern skewing to the anodal gamma fraction in SPE, is indicative for IgG4-RD. Highly sensitive and specific isoelectric focusing assay is helpful in differentiating polyclonal IgG4-RD from other gammopathies.

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Glial fibrillary acidic protein antibodies: a spectacular neuronal immunofluorescence pattern with important clinical implications

To the Editor,
Autoantibodies against intracellular antigen, glial fibrillary acidic protein (GFAP), were recently described in 16 patients presenting with meningoencephalomyelitis; a disease entity termed GFAP astrocytopathy.[1] To date, the presence of these antibodies has been described in a small cohort with heterogeneous clinical presentations.[2,3] Currently, no specific clinical or histological diagnostic criteria exist for GFAP astrocytopathy. Therefore, the presence of antibodies directed against GFAP are critical for diagnosis of this rare and emerging clinical entity.

Eight GFAP isomers have been described, with GFAP-α being the most common autoantibody target.[1,4] Laboratory methods for detection of GFAP-specific IgG include staining of neuronal tissues by either indirect immunofluorescence (IIF) or immunohistochemical methods while transfected human embryonic kidney 293 (HEK293) cell-based assay (CBA) or Western blot (WB) are used as confirmatory assays. The striking fibrillary staining pattern in the cerebellar tissue is characteristic for these autoantibodies, and when identified