BRAF and *NRAS* mutations in circulating Langerhans-like CD1a$^+$ cells in a patient with pulmonary Langerhans' cell histiocytosis

To the Editor:

Pulmonary Langerhans' cell histiocytosis (PLCH) presents as accumulation of Langerhans' cells and other langerin-expressing dendritic cells (LCH cells) in the lungs that cause bilateral nodules and cavities, which are generally restricted to the upper lung fields of adult cigarette smokers [1–3]. The PLCH lung nodules appear to be the origin of the cavities, which could also have a cyst-like appearance that gives rise to a differential diagnosis that includes both cavitary and cystic lung diseases [3]. A characteristic histopathologic feature of PLCH is destruction of the wall of distal airways by infiltration of LCH cells [3]. Given the cellular aetiology, it has been proposed that LCH, and specifically PLCH, might represent a neoplastic or reactive condition [4, 5]. Currently, the diagnostic criteria of LCH include the demonstration of CD1a- and CD207-positive LCH cells in the LCH lesions [6]. As a lipid-presenting molecule, CD1a is abundantly expressed on langerin-expressing cells and accumulates in Birbeck granules, where it colocalises with langerin [6]. The *BRAF* mutations in LCH, including PLCH cases, were detected in 38% to 64% of LCH lesions in two independent studies [7, 8]. Recently, Mourah *et al.* [9] presented the remarkable finding that *NRAS*, in addition to *BRAF* mutations, occur in PLCH lesions. The findings of an abnormal cell and an oncogenic mutation are consistent with the proposal that LCH is a neoplastic disease.

We hypothesised that if Langerhans' cells were indeed neoplastic, and thus, similar to other cancers, circulating tumour cells and DNA could be isolated and might aid in diagnosis and treatment. Circulating tumour cells accompany tumour invasion of the bloodstream. Circulating cell-free tumour DNA (cfDNA) containing oncogenetic mutations is under investigation as a specific biomarker for the diagnosis and monitoring of patients with different cancer types [10]. Expanding knowledge of molecular abnormalities that drive human cancers offers the promise of personalised therapies focused on particular genetic lesions. To date, there is no evidence showing *BRAF* and *NRAS* mutations in circulating Langerhans-like CD1a$^+$ cells and DNA of PLCH patients. Here, we used anti-CD1a antibody to identify circulating Langerhans-like cells by fluorescence-activated cell sorting (FACS). In addition, we evaluated mutations in circulating LCH cells in peripheral blood as well as plasma, as biomarkers in PLCH.

The conditions PLCH and lymphangioleiomyomatosis (LAM) are two rare neoplastic lung diseases of unknown aetiology and different pathogeneses [4, 11]. As a slowly progressive multisystemic disease, LAM primarily affects women of reproductive age, and is characterised by cystic lung destruction. Small clusters of smooth muscle-like cells (LAM cells) can be found in the walls of the cysts and in the pulmonary interstitium [11]. These LAM cells are unique to the disease and contain mutations in the tumour suppressor genes, tuberous sclerosis complex 1 (*TSC1)* or *TSC2* [12]. Circulating LAM cells, which have been isolated based on cell surface markers and cell density [13], have been demonstrated in patients with sporadic LAM and those with TSC [14]. Detection of circulating cells is significantly reduced in patients treated with sirolimus (rapamycin), an inhibitor of the mechanistic target of rapamycin (mTOR).

Patients who were diagnosed with PLCH or LAM were seen at the National Institutes of Health Clinical Research Center in protocols (95-H-0186, 96-H-0100) approved by the National Heart, Lung, and Blood

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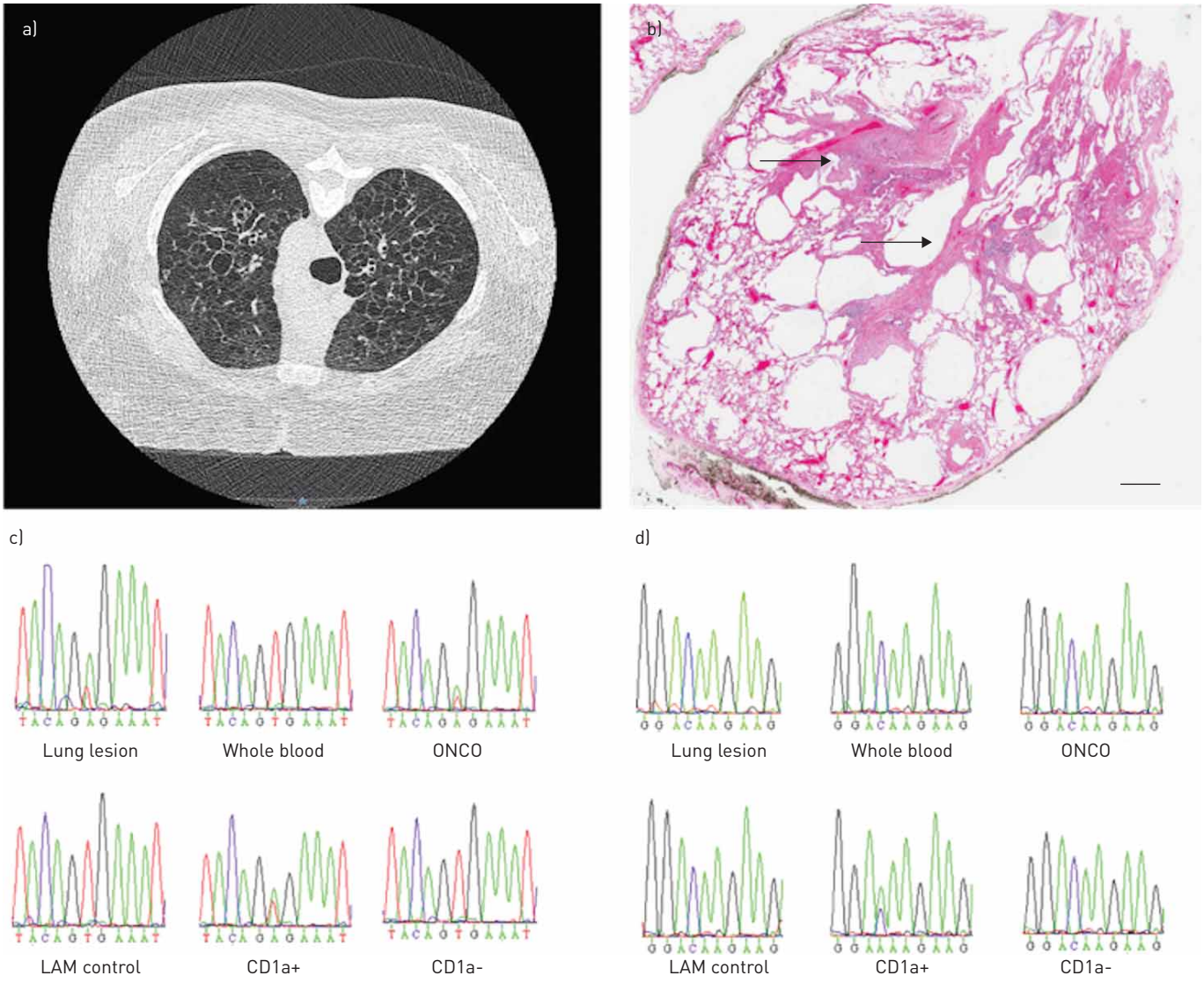

FIGURE 1 a) Isolated Pulmonary Langerhans cell histiocytosis (PLCH) in a 53-year-old-woman. Pulmonary cysts were seen on computed tomography (CT) scans. b) A lung biopsy specimen from the same patient with PLCH under low-magnification, showing enlarged airspaces, and stellate centrilobular scars and cysts. The arrows indicate two larger scars. Scale bar=1 mm. c) *BRAF-V600E* mutation in lung lesion and circulating cells from the same patient. A sequence electropherogram from the same patient demonstrates *BRAF-V600E* mutations in a lung lesion, enriched with OncoQuick (Greiner Bio-One GmbH, Frickenhausen, Germany) and $CD1a^+$ cell fractions. d) *NRAS* mutation in circulating cells from the same patient. A sequence electropherogram from circulating cells of this patient demonstrates a *NRAS* mutation in $CD1a^+$ cell fractions.

Research Institutional Review Board. The diagnosis of PLCH was based on clinical, radiographic, lung biopsy and histopathologic criteria (figure 1a, b). Cells from blood were sorted on the basis of cell surface markers that have been shown to identify LAM cells (CD235a, CD45) or Langerhans-like $CD1a^+$ cells (CD1a) [6, 13]. Mutations in exon 15 of the *BRAF* gene and exon 3 of the *NRAS* gene were analysed by polymerase chain reaction (PCR)-based, direct sequencing using isolated DNA from each cell fraction. The cell and PCR-based method identified *BRAF* and *NRAS* mutations in circulating cells.

When it occurs in internal organs, LCH might result from mutations in stem cells in bone marrow or circulating cells [15]. Milne *et al.* [16] reported a developmental pathway leading to the formation of human Langerhans' cells from circulating $CD14^-$ and $CD1C^+$ dendritic cell precursors in peripheral blood. In other studies, Berres *et al.* [17] found that patients with multisystemic LCH carried the *BRAF* mutation in circulating $CD11c^+/CD14^+$ cell fractions as well as in bone marrow $CD34^+$ progenitor cells. Kobayashi and Tojo [18] evaluated the *BRAF* mutation in cfDNA from eight LCH patients, and reported only three multisystemic LCH patients testing positive for *BRAF-V600E*. Here, we demonstrate the *BRAF-V600E* mutation in lung lesions from a late-stage isolated PLCH patient with diagnosis restricted to the lung. Furthermore, the same mutation was found in circulating LCH cells in an OncoQuick (Greiner Bio-One

GmbH, Frickenhausen, Germany) density gradient-enriched cell fraction and a FACS-isolated $CD1a^+$ cell fraction. The mutation was not found in the circulating LAM cell population ($CD45^-/CD235^{+/-}$) (figure 1c). Moreover, we detected the *NRAS* mutation in circulating cells from the same PLCH patient in the $CD1a^+$ cell fraction (figure 1d), but not in the OncoQuick (Greiner Bio-One GmbH) density gradient-enriched cell faction or in the PLCH lung lesion. The reason for this finding could be, as MOURAH *et al.* [9] pointed out, that *BRAF* and *NRAS* mutations could be observed from different clones of lung lesions. Moreover, the allele frequency of the *NRAS* mutation was often low. The *BRAF* and *NRAS* mutations were not found in cfDNA, suggesting that in PLCH, circulating cells might have higher sensitivity than cfDNA for the detection of both *BRAF* and *NRAS* mutations. Furthermore, *BRAF* and *NRAS* mutations were not identified in circulating cells from two LAM patients, indicating that these two point mutations could be considered as evidence, consistent with a PLCH diagnosis.

Isolated PLCH is rare and its pathogenesis is poorly defined; however, cigarette smoke is seen as a risk factor for disease progression in adults [1]. Recurrence of PLCH after double lung transplantation is consistent with a non-pulmonary source of LCH cells [19]. Our results also suggested that *BRAF* and *NRAS* mutations could come from different sources.

The *BRAF* gene is a member of the *RAF* family of serine threonine kinases that contributes to *RAS-RAF-MAPK* signalling [20, 21]. Somatic-activating *BRAF* gene mutations are observed in a range of cancers, including melanoma, colorectal and lung cancer [21]. Among the *BRAF* mutations, V600E is by far the most common, and it results in a constitutively active protein. It has been proposed that unabated activation of the *MEK-ERK* pathway contributes to dysregulated cell proliferation, survival and ultimately malignant progression [20]. Here, we confirmed that LCH-derived *BRAF* mutations could be enriched in $CD1a^+$ fractions of circulating cells, consistent with the classification of PLCH as a neoplastic disorder.

In addition, we also detected NRAS, as might be expected based on the study by MOURAH *et al.* [9]. The *NRAS* gene is included in the *RAS family* of oncogenes, which consists of three members: *HRAS, KRAS* and *NRAS* [22]. Each isoform of *RAS* displays preferential coupling to particular cancer types. The *NRAS* mutations were present in 25–30% of melanomas [23], in 0.7% of patients with lung cancer [24], and in 10.3% of patients with myeloid leukaemia [25].

In summary, we report a procedure for the detection of *BRAF* and *NRAS* mutations in circulating Langerhans-like $CD1a^+$ cells. The procedure might help to differentiate PLCH from other cavitary and cystic lung diseases.

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