Clinical responses observed in patients treated with BRAF inhibitors do not support the suggestion of intra-patient BRAF heterogeneity as all metastases have a uniform initial metabolic response to BRAF inhibition assessed using FDG-PET imaging (McArthur *et al*, 2012), and all resistant lesions resected from patients still contain mutant *BRAF* (McArthur *et al*, 2011; Poulikakos *et al*, 2011; Van Allen *et al*, 2013).

Further clinical studies are required to examine the issue of intra-patient discordance of *BRAF*. Carefully assigning primary melanomas as culprit lesions, and using accurate *BRAF* testing methods with adequate tumour cell content would be the requirements to underpin the data.

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CONFLICT OF INTEREST

AMM has received honoraria from Roche and travel support from Roche and GlaxoSmithKline (GSK). JSW declares no conflict of interest. GVL has been a consultant for Roche, Bristol-Myers Squibb, GSK and Novartis, and has received honoraria and travel support from Roche. RAS has been a consultant for Roche and GSK, and has received honoraria from Abbott Molecular.

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Reply: Intra-patient heterogeneity of BRAF mutation status: fact or fiction?

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We thank Menzies *et al* (2014b) for their interest in our work and their detailed and informative remarks that extend what we discussed in our paper. They are concerned that our findings of an unexpected high percentage of heterogeneity reflect methodical problems of mutation detection rather than tumour biology. In contrast, our main worry is that acknowledged and widely used diagnostic techniques could exclude a significant percentage of patients from BRAF inhibitor therapy despite the presence of mutated metastases. Indeed, our study was initiated because we could not believe in the intrapatient heterogeneity even though we like other groups (Houben *et al*, 2004) were occasionally getting divergent results when retesting new metastases from patients. We will try to explain in our reply why we do not believe that there are 'easy' explanations such as lack of sensitivity, low tumour content in samples studied and higher sensitivity of immunohistochemical analyses compared with direct mutation detection.

We are aware that our findings could be due to sensitivity of our testing methods. The suggested approach of immunohistochemistry (IHC), however, will not suffice to detect BRAF mutations. Indeed a substantial patient population will be missed as we and others have shown that rare BRAF mutations are not (V600K, V600D, L597S, V600DK601del, V600R) or not always detected by IHC (Skorokhod *et al*, 2012; Heinzerling *et al*, 2013).

Similarly, the COBAS test does not reliably detect rare mutations (Heinzerling *et al*, 2013). Rare mutations have been described in up to 20% of BRAF-mutated patients by your group and others (Beadling *et al*, 2011; Long *et al*, 2011; Dahlman *et al*, 2012) and it is crucial to detect them as these patients respond to therapy with BRAF inhibitor (Chapman *et al*, 2011; Klein *et al*, 2013). Thus, even though possibly the intrapatient heterogeneity might be lower in the published IHC study by Menzies *et al* (2014a) using IHC as only detection technique would exclude patients with actionable mutations from effective treatment with a BRAF inhibitor. Furthermore, discordance rates of course also depend on the number of samples tested. And even the study with lowest rates of heterogeneity only using paired samples of primary tumour and one metastatic lesion found heterogeneity in some patients with concordant results in 90.9% (Boursault *et al*, 2013). It is likely that the rate of heterogeneity is higher when testing more samples per patient (up to 13 in our studies) and as shown by Colombino depends on the type of metastases with highest rates of 24% heterogeneity for skin metastases (overall discordance rate: 15%; Colombino *et al*, 2012). Furthermore, in our article we show intratumoural heterogeneity of the immunohistochemical BRAFV600 staining, a finding that has been confirmed by other groups using molecular methods (Lin *et al*, 2011; Yancovitz *et al*, 2012). In addition,

heterogeneity has been detected not only between primary tumour and metastases, which could be explained by multiple primaries or occult primaries, but also between metastases, and this explanation certainly could not account for the rates of discordance seen. In our hands, we routinely use IHC as an important additional method for BRAF mutation screening.

Test sensitivity has been described differentially for the various testing methods (Lade-Keller *et al.*, 2013). It was shown, however, that in samples with at least 10% tumour cell content 100% consensus was achieved between five different methods: the COBAS test, Sanger sequencing, pyrosequencing, TaqMan-based allele-specific PCR and competitive amplification of differentially melting amplicons. The sensitivity of pyrosequencing has previously been tested using DNA dilutions mixing heterogenous V600E tumour with normal lymphocytes and found to be highly accurate even if tumour content was only 20% (Spittle *et al.*, 2007). In this study we have microdissected the tumour area from the tissue sections that yielded a tumour content of >75% as described previously (Heinzerling *et al.*, 2013). Accounting for the heterozygous presence of the mutated gene in most tumours (Sigalotti *et al.*, 2011) and the presence of stromal elements this is well above the detection limit.

In the clinical context, the majority of patients respond to BRAF inhibitors and mostly, metastases uniformly regress and then progress again once resistance is acquired. We even saw this pattern in one discordant patient (patient #3). However, besides the specific inhibitory effect of BRAF inhibitors on BRAF V600-mutated cells, relevant immunological effects of BRAF inhibitors are increasingly becoming apparent. A reversion of immunosuppression by vemurafenib with a decrease of immunosuppressant myeloid-derived suppressor cells in response to treatment has been reported (Schilling *et al.*, 2013) as well as a restoration of compromised dendritic cell function (Ott *et al.*, 2013). Similarly, an analysis of lymphocyte counts in peripheral blood has shown a differential influence of vemurafenib and dabrafenib (Schilling *et al.*, 2014). Furthermore, *in vitro* treatment with BRAF inhibitors lead to an increased expression of melanocyte differentiation antigens conferring enhanced antigen-specific recognition by cytotoxic T lymphocytes without compromising lymphocyte function (Boni *et al.*, 2010). Thus, potentially the response to BRAF inhibitors could be partially mediated immunologically, which is backed by the finding of a marked T-cell infiltration induced by BRAF inhibitor therapy *in vivo* in melanoma patients (Wilmott *et al.*, 2012). This could implicate that even wild-type metastases could respond to therapy with BRAF inhibitors. However, these hypotheses still need to be further evaluated.

Until now, mutation results from different tumour samples of one patient may differ for various reasons that could lead to exclusion of the patient from effective BRAF therapy. As stated in the conclusion of our paper and in the letter of Menzies *et al.* (2014b), the role of heterogeneity in testing needs to be further investigated because it has profound clinical consequences; and as shown by our publication, it quite surprisingly relates to a substantial subset of patients tested by acknowledged diagnostic methods.

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