Tumor cells may circulate in medullary thyroid cancer patients independently of serum calcitonin

Dear Editor,

Early detection of tumor relapse is a major issue in patients with medullary thyroid carcinoma. Calcitonin has been reported as a sensitive and accurate marker for recurrence of medullary thyroid carcinoma after thyroidectomy. Recent evidence nevertheless reveals pitfalls in calcitonin immunoassays due to the presence of heterophilic antibodies or macroaggregates (i.e. falsely increased values or macrocalcitonin) (Alves et al. 2016). Calcitonin can also remain undetectable despite metastasis of rare tumor cells in thyroidectomized patients. In this context, we designed a sensitive and specific technique to identify calcitonin-positive circulating tumor cells (CTC) in medullary thyroid carcinoma. We demonstrate that calcitonin-positive CTCs are present in the peripheral blood of medullary carcinoma patients following complete thyroidectomy. Unexpectedly, the presence of CTCs could be identified up to 12 years after surgery even in the absence of high levels of serum calcitonin.

Incidence of thyroid cancer significantly increased in the past several decades (Kuo et al. 2016). Tumors most frequently arise from follicular epithelial cells to generate papillary, follicular or anaplastic thyroid cancers. Medullary thyroid carcinoma (MTC) initiates in neuroendocrine C cells accounting for approximately 4–5% of all thyroid cancers (Xing 2013). Since MTCs are unresponsive to radioiodine therapy, surgical resection of the tumor is the first-line treatment. With 10-year survival rates of about 75%, thyroidectomy is generally the most adequate therapeutic option. The main issue in MTC management is prediction of tumor relapse. With 10-year survival rates of about 75%, thyroidectomy is generally the most adequate therapeutic option. The main issue in MTC management is prediction of tumor relapse. The serum concentration of calcitonin is a specific and cost-effective biomarker that adequately predicts tumor relapse or metastasis. Indeed, the doubling time of serum calcitonin is a reliable prognostic marker used in routine management of MTC (Ito et al. 2016). Recent evidence has nevertheless challenged calcitonin-based immunoassays due to the presence of heterophilic antibodies in patient serum or cross-reactivity with procalcitonin and related peptides (Alves et al. 2016). These issues should be solved to improve post-operative prognosis. When calcitonin levels exceed 500pg/mL, distant metastases are almost always identified by radiographic imaging. In contrast, when serum calcitonin is low, the risk of persistent or recurrent residual disease is low. A challenge in decision-making occurs in presence of intermediate calcitonin levels (150pg/mL) or when concentrations are persistently increased but unchanged over time. In these conditions, local or distant metastases are difficult to detect by radiographic imaging and require additional and often expensive tests during follow-up. In this context, we investigated another approach based on the identification of thyroid-specific CTCs. CTCs are cells that detach from the primary or metastatic tumor and intravasate into the blood stream (Yu et al. 2011). Since CTCs travel in the blood to develop a secondary tumor at a distant site, their frequency can be used as a prognostic marker for relapse. Currently, main issues in CTC enumeration pertain to their extreme scarcity compared to blood cells and to phenotypic changes (epithelial-to-mesenchymal transition (EMT)). A series of tests have been developed based on cell size fractionation, microfluidics or antigen capture. In particular, the CellSearch (Veridex) is a FDA-validated system that enumerates CTCs upon capture of EpCAM-positive epithelial cells in whole blood. If exceeding a defined threshold, CTC levels have been demonstrated to worsen prognosis of the situation (Yu et al. 2011). The principle of the technique is based on automated sorting of CTCs with an epithelial marker (EpCAM) and subsequent detection of CD45-negative, cytokeratin-positive nucleated cells. To identify CTCs
in thyroid cancer, a series of 15 patients was analyzed by CellSearch (study #2014/93 approved by the Ethics Committee of the CHU University Hospital, Liège, Belgium). In the majority of cases (60%, 9 out of 15), no CTC was identified (Fig. 1A, B and Supplementary Table 1, see section on supplementary data given at the end of this article). Four subjects out of 15 (27%) had 1 CTC in 7.5 mL of blood. The two remaining patients (2 out of 15) with medullary and papillary thyroid cancers displaying lung and lymph node metastases had 2 and 7 CTCs, respectively. As controls, CTCs were enumerated in cancers associated with high (small-cell lung cancer (SCLC)) and low (non-small-cell lung cancer (NSCLC)) cell counts. In accordance with previous studies (Krebs et al. 2011, Hou et al. 2012), CTCs were detected in all SCLC cases (n=6) and in most NSCLC (n=41 out of 67) cases (Fig. 1B and Supplementary Table 1). These data thus indicate that EpCAM-positive CTCs are undetectable in most thyroid cancer patients validating and extending previous observations (Yu et al. 2016). Therefore, we set up another technique based on cell filtration (ScreenCell device) and calcitonin expression to identify CTCs in MTC. The reactivity of a FDA-approved antibody used in routine diagnosis (SP17) was first evaluated on three cell lines pertaining to the major thyroid cancer subtypes: TT (medullary), TPC-1 (papillary) and C643 (anaplastic). Immunofluorescence analysis showed that only TT cells expressed calcitonin, as expected (Fig. 1C and Supplementary Fig. 1). Immunohistochemistry of a thyroid biopsy further indicated that parafollicular C cells specifically stained positive for calcitonin (Supplementary Fig. 2). These experiments thus validate the specificity of the anti-calcitonin SP17 antibody.

Using the ScreenCell device, CTCs were isolated from peripheral blood of a patient with MTC and stained with SP17. Figure 1D illustrates a typical fluorescence scan of CTCs expressing calcitonin in the cytoplasm and containing a DAPI-stained nucleus (blue arrows). A major criterion to distinguish CTCs from leucocytes is their larger diameter (at least three-fold) that does not allow filter pore bypass. Although the filter pores yielded some background autofluorescence, the specificity of labeling was demonstrated by the presence of another cell lacking calcitonin expression (see * on Fig. 1D) and by spike-in experiments (Supplementary Fig. 3). In contrast, no calcitonin-positive CTCs could be detected in peripheral blood from four healthy volunteers (Supplementary Fig. 4).

Using these optimized experimental conditions, the presence of calcitonin-positive CTCs was investigated in a series of subjects with MTC. We selected nine patients at different TNM stages having undergone complete thyroidectomy and, except for #9, lacking any evidence of other cancer (Table 1 for clinical and pathological characteristics). The calcitonin concentrations in the serum were above the basal level of the assay (i.e. 10 pg/mL) and ranged from 48 to 10,600 pg/mL, further validating the diagnosis. Calcitonin-positive CTCs were identified in all patients (from 1 to 7), except one (#1). Interestingly, the CTC counts did not correlate with TNM classification as illustrated for example by patient #1 at pT3N1bM1 lacking any detectable calcitonin-positive circulating cell while 5 CTCs were scored in subject #7 at pT1N1bMx. Finally, the serum calcitonin concentrations did not correlate with CTC counts ($R^2=0.11272$, Fig. 1E). In particular, the calcitonin levels were low in patients #5, #7 and #8 (70, 48 and 82 pg/mL) while CTC counts were high (n=4, 5 and 7), respectively.

We have thus set up a technique that combines the ScreenCell device and calcitonin labeling that very specifically identifies CTCs in MTC patients even after thyroidectomy. Compared to the FDA-cleared CellSearch, our experimental protocol has a series of advantages. First, the CellSearch enumerates CTCs by cell counting. In accordance with previous studies (et al. 2016), we set n=6 for clinical and pathological correlation. In contrast, our method is able to identify calcitonin-positive CTCs in thyroidectomized patients considered to be in persistent disease. Secondly, calcitonin labeling specifically identifies CTCs derived from MTC excluding any other neoplasm such as breast cancer (patient #9 in this study). Third, gentle cell filtration by ScreenCell is also compatible with microemboli that cannot be scored by CellSearch. Fourth, the protocol does not require any expensive equipment in addition to filtration columns. The ScreenCell device and calcitonin labeling approach also has some disadvantages. Although visual inspection very specifically reveals fluorescent cells expressing calcitonin, validation of CTC phenotype requires the intervention of a trained pathologist. The technique also requires further automation to be compatible with routine diagnosis. Another limitation of the technique is that blood samples should be analyzed fresh or at least within a maximum of 92 h. Despite these drawbacks, the ScreenCell device and calcitonin labeling methodology
can, as described, readily be set up in standard conditions of routine diagnosis.

Although the series of samples are limited, this report highlighted an unexpected and interesting observation: relatively high CTC counts can be detected despite low calcitonin levels in the serum of three patients (#5, #7 and #8). Since CTC numbers correlate with the probability of tumor relapse, there is an intrinsic risk that prognosis of these subjects will worsen in the near future. Careful follow-up of these patients and further validation in larger cohorts will confirm this observation. This is particularly important because follow-up periods in our series are longer in the group of low CTC count (especially in cases 1, 2 and 4) compared to the group of high CTC count (cases 5–9). Therefore, overall survival or disease-free survival may show poorer in high CTC count group than in low CTC count group. Nevertheless, we believe that the presence of calcitonin-positive CTCs in the absence of significant levels of serum calcitonin should be considered to initiate additional tests during follow-up. There is indeed growing evidence that the presence of CTC correlates with poorer prognosis (Yu et al. 2016). Further follow-up experiments are required to assess whether prognosis depends on calcitonin-positive CTC counts. Since neither radiotherapy nor chemotherapy has demonstrated durable objective responses in patients with advanced MTC, early identification of tumor relapse is predicted to increase overall survival.
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Supplementary data
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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

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Author contribution statement
S. N. S. performed the lab experiments and analyzed raw data. A. B., E. H., M. C. and R. L. organized trial and analyzed clinical data. M. C. contributed to patient recruitment and ethics. A. B., E. H. and L. W. conceived and designed the experiments. All co-authors contributed to writing and critical reading of the manuscript.

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