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Detection and Clinical Significance of Circulating Tumor Cells in Colorectal Cancer—20 Years of Progress

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Circulating tumor cells (CTC) may be defined as tumor- or metastasis-derived cells that are present in the bloodstream. The CTC pool in colorectal cancer (CRC) patients may include not only epithelial tumor cells, but also tumor cells undergoing epithelial–mesenchymal transition (EMT) and tumor stem cells. A significant number of patients diagnosed with early stage CRC subsequently relapse with recurrent or metastatic disease despite undergoing "curative" resection of their primary tumor. This suggests that an occult metastatic disease process was already underway, with viable tumor cells being shed from the primary tumor site, at least some of which have proliferative and metastatic potential and the ability to survive in the bloodstream. Such tumor cells are considered to be responsible for disease relapse in these patients. Their detection in peripheral blood at the time of diagnosis or after resection of the primary tumor may identify those early-stage patients who are at risk of developing recurrent or metastatic disease and who would benefit from adjuvant therapy. CTC may also be a useful adjunct to radiological assessment of tumor response to therapy. Over the last 20 years many approaches have been developed for the isolation and characterization of CTC. However, none of these methods can be considered the gold standard for detection of the entire pool of CTC. Recently our group has developed novel unbiased inertial microfluidics to enrich for CTC, followed by identification of CTC by imaging flow cytometry. Here, we provide a review of progress on CTC detection and clinical significance over the last 20 years.

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INTRODUCTION

CRC is the second most common cancer in Australia, with over 15,000 cases diagnosed in 2012, and is the second most common cause of cancer-related deaths (1). In the United States CRC is the third most common cancer and the third leading cause of cancer death in men and women (2). Patients diagnosed with early stage CRC undergo surgical tumor resection with curative intent, yet 20–30% of these patients suffer recurrent or metastatic disease within 5 years of surgery. This suggests that an occult metastatic disease process was already underway (3), or that viable tumor cells with proliferative and metastatic potential had been shed into the bloodstream from the primary tumor site during surgical resection to cause subsequent disease relapse in these patients (4,5). These cells are termed circulating tumor cells (CTC), broadly defined as tumor- or metastasis-derived cells that are present in the bloodstream. In particular, detection of CTC can identify patients with early-stage disease who are at risk of developing recurrent or metastatic disease (4,6) and who would thus more likely benefit from adjuvant therapy after resection of the primary tumor. Although much effort has been directed at the detection of CTCs (reviewed in [7–9]), large prospective clinical trials have yet to be completed to determine prognostic significance for monitoring minimal residual tumor cells to assess response to therapy in advanced disease as well as in the adjuvant setting.

PRESENCE OF CTCs AS AN ADJUNCT TO STAGING

A major determinant of patient prognosis is the stage at which the cancer is diagnosed, because surgery is considered curative in up to 70% of early-stage cases. Screening programs have helped

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with early diagnosis and intervention, but for those not participating in such programs some 12–25% of CRC patients still present with advanced (stage IV) disease (10). Up to 20% of patients diagnosed with early-stage CRC (stage I or II) and up to 30% with regional spread to lymph nodes or adjacent organs (stage III) have relapsed by 5 years after "curative" surgery (2,11). Furthermore, the introduction of laparoscopic surgery for CRC has not altered the 5-year survival rates after curative surgery compared with open surgery (12). Stage III tumors are a heterogeneous group with respect to outcome: lymph node involvement is of limited reliability in predicting recurrence or the need for adjuvant chemotherapy. Furthermore, a significant proportion of patients still show recurrent disease despite receiving adjuvant chemotherapy. Current tumor staging techniques of histology and radiological imaging are not sensitive enough to detect micrometastases or early tumor cell dissemination, events key to developing metastatic disease. The presence of CTCs may be indicative of a micrometastatic process involving distant organs and may have prognostic implications independent of established staging factors such as the extent of lymph node involvement. We have previously shown, using immunobead reverse-transcription (RT)-PCR, that in stage III patients, detection of epithelial cells in peripheral blood in 13/31 (42%) patients correlated with shorter disease-free survival (DFS) (hazard ratio [HR] 2.8, 95% confidence interval [CI] 1.169–6.716), suggesting that the presence of CTC has the potential to more accurately stratify stage III patients into different prognostic groups (6). Furthermore, our analysis of disseminated tumor cells (DTC) in postresection peritoneal lavage samples showed that stage I and II patients who were positive for DTC showed a significantly poorer outcome (HR for DFS, 6.2; 95% CI, 1.9–19.6), and this was independent of other established risk factors (13). Although adjuvant chemotherapy is offered to stage III patients to eradicate potentially existing micro-metastases, the indication for such treatment in stage II disease is less certain, with only small clinical benefits shown in clinical trials (14). The dilemma is finding a balance between the benefit of therapy, which may be incremental, and risk of harm, and to that end biomarkers, such as CTCs that correlate with impending recurrent or metastatic disease, are much needed.

ENRICHMENT AND DETECTION OF CTCs

The isolation and molecular analysis of CTCs in peripheral blood is one of the most promising approaches to identifying disseminated disease but there are challenges in identifying unique tumor physical characteristics and tumor-specific phenotypic or molecular markers. CTCs are detectable at very low numbers relative to white blood cells (in the order of 1 CTC in 10^7) (15) but may be present at much higher numbers in metastatic cancer patients. The large majority of clinical studies available to date relied solely on the number of CTCs in blood samples determined on the basis of isolation of epithelial tumor cells by monoclonal antibody (Ber-EP4) targeting epithelial cell adhesion molecule (EpCAM), originally selected by our group for the immunomagnetic isolation of carcinoma cells from blood (16). EpCAM, as the target for antibody-labeled magnetic microbeads for this purpose, was later commercialized as Epithelial Enrich™ (Dynal Biotech). EpCAM is also used as the target for CTC capture in the CellSearch™ system (Veridex LLC), approved by the Food and Drug Administration (FDA) for monitoring response to treatment in breast, colon and prostate cancer (17–20). In the first prospective multicenter study in metastatic CRC using the CellSearch™ System, CTCs were enumerated in a 7.5-mL blood sample from 430 patients. The results showed that patients in whom ≥3 CTC/7.5 mL blood were detected had shorter median and overall survival (OS) compared with patients with <3 CTCs ($P < 0.0001$), and these differences persisted at follow-up time points after therapy. They concluded that the number of CTCs was an independent predictor of DFS and OS in metastatic CRC and provided prognostic information in addition to that of imaging studies (19). Since these early reports, studies on the detection of CTC using immunoaffinity-based enrichment strategies have abounded and have generally shown that CTC levels of >1 cell/mL in peripheral blood (PB) correlate with poor prognosis. A metaanalysis of 36 studies (3,094 patients) that included PB, mesenteric portal blood (MPB), or bone marrow (BM) sampling showed that detection of CTCs in PB, but not in MPB or BM, correlated with poor prognosis in CRC (HR for DFS 3.06, 95% CI 1.74–5.38). The inclusion criteria were the use of any form of PCR and/or immunologic or flow cytometric detection techniques (21). A more recent metaanalysis of 1,329 patients with metastatic CRC showed that OS (HR, 2.47; 95% CI 1.74–3.51) and DFS (HR, 2.07; 95% CI 1.44–2.98) were shorter in patients with CTCs. Of note, the main inclusion criteria for the studies was that the presence of CTC had to be detected by methods that relied on monoclonal antibodies to EpCAM or cancer-specific antigens (22). Furthermore, most of the studies described in this review and the 12 studies included in the metaanalysis by Sergeant *et al.* (2008) (7) used expression of cytokeratin 19 (*CK19*), *CK20* or carcinoembryonic antigen (*CEA*) to identify CTCs, markers that we and others have reported show a high background expression in blood samples from patients with inflammatory bowel disease and result in an unacceptably high rate of false positives (6,7,13,23). Despite these shortcomings, CTC levels enumerated using the CellSearch system showed promise as an early treatment response marker in metastatic breast cancer and could be useful in clinical trials alone or in addition to radiological assessment of response (24). A change in CTC load is also actively being investigated as a surrogate marker for the assessment of therapeutic efficacy: in a study of 90 stage III colon cancer patients receiving adjuvant oxali-

platin with fluorouracil and folinic acid (FOLFOX) chemotherapy the persistent presence of CTCs postchemotherapy was shown to be an effective marker for determining clinical outcome (HR for DFS, 6.27; 95% CI, 2.44–16.12 (25). Interestingly, in a clinical trial of a 4-drug regimen in advanced CRC, patients with high CTC numbers (detected by CellSearch) survived longer than expected compared with historical controls, whereas patients with low CTCs gained no extra benefit. This may identify patients who could be spared high-toxicity regimens (26). In a recent study including 239 patients with nonmetastatic potentially curable CRC, the rate of CTC positivity correlated with increasing stage. In this study multivariate analysis showed that CTC numbers (≥1 CTC/mL blood) were the strongest prognostic factor in nonmetastatic patients (stage I, II, III); HR, 5.5; 95% CI, 2.3–13.6), with similar results for the total study group of 287 patients (HR, 5.6; 95% CI, 2.6–12.0) (27).Although the detection of CTCs as a prognostic marker has been well established, CTC enumeration has yet to be accepted into the clinic in guiding treatment decisions for individual cancer patients. We and others have shown that not all patients positive for CTC suffer disease progression, and conversely disease progression does occur in some patients negative for CTCs (4,13,19,28). Nevertheless, CTCs may be detected much earlier than current standard-of-care imaging (computed tomography [CT] or positron emission tomography [PET]-CT) for metastases (reviewed in [29]). In a study of advanced CRC (n = 451) the CTC count (by CellSearch) before and during treatment was an independent prognostic marker for PFS and OS (30). There was also a statistically significant correlation between CTC levels and tumor response according to CT imaging; however, there were still issues of sensitivity and specificity, because only 4 of 20 patients (20%) with progressive disease had high CTC levels at 1–2 wks postbaseline (30). Hence further research is needed to improve the sensitivity and specificity of CTC detection. For instance, EpCAM expression was found to be in the order of 10-fold less on CTCs compared with primary and metastatic tissues, suggesting that EpCAM expression is dependent on the local microenvironment and is downregulated in disseminated cells (31). This is quite probably the reason why CTCs were undetected by the CellSearch system in a significant proportion of patients with colorectal (32) and other cancers (reviewed in [33]). Downregulation of EpCAM has also been reported in DTCs in bone marrow in breast cancer patients following adjuvant therapy (34) and was found to be the reason for failure to detect any CTCs in a murine breast cancer xenograft model (35). Furthermore, tumor cells that have gained entry to the bloodstream interact with platelets, during which platelet-derived transforming growth factor β (TGFβ) contributes to epithelial–mesenchymal transition (EMT) in the tumor cells via activation of the TGFβ/Smad and nuclear factor (NF)-κB pathways (36). EMT+ cells possess a motile, more stemlike phenotype expressing N-cadherin, vimentin and fibronectin (37,38), with concomitant loss of epithelial cell adhesion molecules such as E-cadherin (39) and EpCAM (35). The EMT phenotype of CTC has been linked to resistance to both chemotherapy and radiation therapy (reviewed in [40]). Methodologies that rely on positive capture of CTC, which have to assume specific criteria such as high expression of EpCAM, are intrinsically flawed, so that the real CTC load is underestimated and stem cell populations of likely high relevance to disease progression are missed (33). On balance, due to the relatively low sensitivity and specificity of the EpCAM-based approach EpCAM-targeted capture strategies cannot still be considered the best approach for CTC isolation (41–43).

THE EMERGING CIRCULATING TUMOR STEM CELL HYPOTHESIS

There is increasing evidence that CTCs comprise a heterogeneous pool of cells that includes epithelial tumor cells, tumor cells undergoing EMT, and tumor stem cells, and they may circulate as single cells or clusters of cells (33,38,42). We believe that it is the stem cells within the circulating tumor cell pool that are responsible for metastatic disease progression: these cells are termed circulating tumor stem cells (CTSC) (33) (Figure 1). This view is bolstered by the presence of stem cell phenotypes CD26+ and CD133+ in CTCs of metastatic CRC patients (44,45) and the finding that increased expression of the exclusive stem cell marker leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) in colorectal tumors indicates a poor prognosis (46,47).

In an attempt to address the issues with positive selection, Iinuma *et al.* (45) used an unbiased approach for CTC isolation and included the stem cell marker CD133 in the identification step. Their multiinstitutional study of 735 CRC patients used mRNA from nonenriched whole blood; they showed that detection of CTCs positive for CEA/CK19/CK20/CD133 expression (RT-PCR) was a significant prognostic factor for poorer DFS and OS in Dukes stage C patients, and in stage B patients with unfavorable pathological features, but not for stage A or stage B patients with otherwise good prognostic features. They concluded that CEA/CK/CD133+ CTC, but not CEA/CK+ CTC, was an independent prognostic factor in patients with Dukes stage B CRC (45). These results also showed a large difference in survival curves for stage C patients, all of whom received adjuvant chemotherapy, suggesting that CEA/CK/CD133+ CTC could be a marker for the presence of treatment-resistant stem cells (41). One caveat is that CD133 is also expressed on endothelial cell progenitors (48), so although not specific for tumor stem cells, CD133 could be a surrogate marker for tumor angiogenesis and tumor progression. This raises the question as to what are the likely markers for detection of CTSCs.

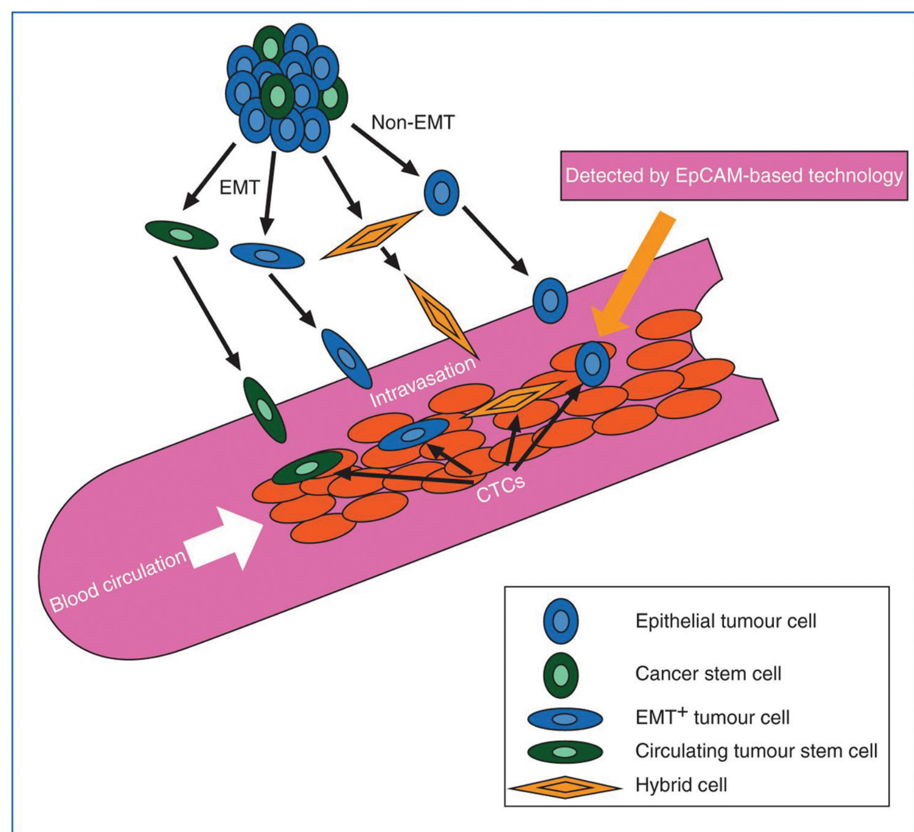

Figure 1. Intravasation of tumor cells into the bloodstream: some at least survive immune destruction and are capable of seeding metastases in distant organs. Hybrid cell refers to a cell transitioning between an epithelial and mesenchymal phenotype. Image reproduced by permission of the European Society of Medical Oncology (Lugano, Switzerland) from reference (33): PK Grover, AG Cummins, TJ Price, IC Roberts-Thomson, and JE Hardingham. Circulating tumour cells: the evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research. *Annals of Oncology*. 2014;25(8):1506–1516.

IDENTIFICATION OF STEM CELL MARKERS FOR CTSC DETECTION

We have tested the differential expression of several stem cell markers such as *LGR5*, olfactomedin 4 (*OLFM4*), achaete-scute complex homolog 2 (*ASCL2*) and B lymphoma Mo-MLV insertion region 1 homolog (*BMI-1*) (49,50) between CRC patients' tumor tissue and their matched normal mucosa. *OLFM4* and *LGR5* were overexpressed ≥2-fold in 18/22 (82%), *ASCL2* in 13/22 (59%) and *BMI-1* in 9/22 (41%) tumors (Figure 2). *OLFM4* has previously been identified as a good marker of intestinal stem cells and is involved in regulation of cell adhesion and organization of the cytoskeleton (50,51). *LGR5*+ crypt base columnar cells have been found to be capable of generating all intestinal epithelial lineages, in both the small intestine and colon (52). Furthermore, single *LGR5*+ cells from the base of small intestinal crypts were found to be capable of initiating growth of crypt-like structures in culture (53). Recently plastin 3 (PLS3) overexpression in CTCs has been reported to induce EMT+ CTCs, and the detection of such cells in a large set of patients with CRC was an independent prognostic factor for PFS and OS; furthermore, the PLS3+ CTCs retained prognostic significance in the Dukes B subset (54). Taken together, these findings suggest that OLFM4, LGR5 and PLS3 may be useful to include in marker panels for the detection of CTSCs. Other markers of CRC stem cells have been proposed but are yet to be validated in the clinic for CTSC detection (55–58).

NEW TECHNIQUES FOR CTC ENRICHMENT AND IDENTIFICATION

Considering the now well-established phenotypical heterogeneity of CTCs, in particular the variation in antigen expression in cancer cells at various stages of EMT (reviewed in [33]), unbiased CTC isolation technologies are now being developed that avoid the use of antibody-based enrichment. Some of these methods exploit physical differences in size, density, deformability or electrical properties between CTCs and blood cells (reviewed in [59]), (9,60). However, methods that rely on the assumption that CTCs are larger than nucleated blood cells are flawed, as tumor cells in different stages of the cell cycle, undergoing apoptosis, or in EMT may be smaller in size (59). In addition, the issue of CTC deformability is controversial, being difficult to characterize and lacking precision (61). Thus, the performance in clinical settings of current physical isolation methods is likely to be lower than predicted from model studies with tumor cell lines. To circumvent the limitations of current CTC isolation platforms relying on either molecular markers or physical characteristics, technologies based on high-throughput automated microscopy of the whole nucleated cell population are desirable, as attested by the development of a number of such commercial systems and clinical services (Epic, RareCyte™, CytoTrack, SRI FASTcell™). However, reliable capture of all tumor cells from a blood sample on the solid substrates to be imaged is required to enable truly unbiased characterization of CTCs based on high-throughput microscopy. To this end, one promising approach makes use of nano-engineered substrates for efficient binding of tumor

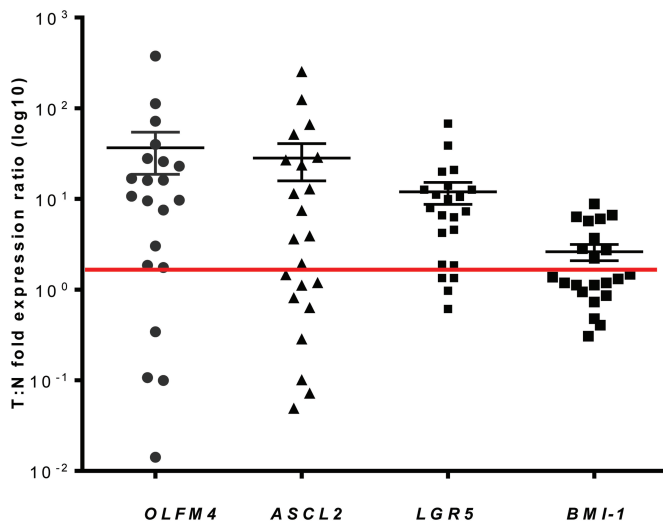

Figure 2. RT-PCR of stem cell markers: tumor versus matched normal (T:N) mucosa expression ratios ($2^{-\Delta\Delta Ct}$) normalized to phosphomannose mutase (PMM1) reference gene expression (*y* axis). Horizontal line shows two-fold overexpression cutoff.

cells (reviewed in [62] and [63]). Recently our group has described the use of nano-roughened polystyrene tissue-culture plates for the capture of CTCs from blood following lysis of red blood cells. The captured cells were stained using fluorophore-tagged antibodies to cytokeratins, CD45 (to preclude detection of white blood cells), vimentin, and DAPI nuclear stain (to exclude cell fragments) and detected using the Operetta automated high-content imaging system (PerkinElmer) (64). This study showed that nanoscale roughness significantly increased the binding of a range of tumor cells (up to 97%), and morphological analysis demonstrated strong adherence of the cells to the nano-rough polystyrene substrates. By using these plates and the Operetta automated high-content analysis system, CTCs were detected in the blood of a stage III CRC patient, demonstrating the feasibility of this method in a clinical setting (64). However, recent technological advances in the field of imaging flow cytometry (IFC) circumvent altogether the need for CTC capture on solid substrates. IFC is an ideal technology because it combines the speed, statistical power and fluorescence sensitivity of flow cytometry with the morphological insights of high-resolution microscopy. A very high throughput of 100,000 particles/second and a false-positive rate of one in a million was recently achieved by use of ultrafast optical imaging technology and self-focusing microfluidic technology (65); this technology has been used to quantify androgen receptor expression and subcellular localization in CTCs of prostate cancer patients (66). Moreover, using the ImageSteam®X Mark II IFC instrument (Amnis), we could detect CTCs in the blood of patients with both early and advanced CRCs. Importantly, unlike conventional flow cytometry, IFC can be used to identify double-positive events (CD45+/CK+) associated with commonly observed CTC/white blood cell aggregates as well as CK+ CTC clusters (Figure 3).Importantly, high-throughput microscopy and IFC can easily be combined with cutting-edge CTC isolation technology. The ability to analyze large volumes of blood is expected to be especially important for nonmetastatic diseases in which the CTC burden is often small. One such approach we are currently investigating relies on the concept of an inertial microfluidic device (67). This microfluidic device has the advantage of being able to process a much larger volume of blood (up to 20 mL) than previously possible, reducing the number of white blood cells in the fraction to be analyzed by 99.9%. The remaining cell fraction is typically stained with anti-human CD45, anti-pan-cytokeratin, anti-E-cadherin and anti-vimentin using our established protocol (64) and analyzed using IFC. Once clinically validated, this highly efficient, unbiased tumor cell isolation technology, together with IFC using appropriate markers including EMT and stem cell markers, has the potential to provide a cost-effective technological solution toward large-scale clinical implementation of CTC-based diagnostic and prognostic strategies, something that has been missing up until now. In the near future CTC, and more specifically CTSC detection and enumeration, should provide a useful adjunct to CT and MRI imaging and perhaps even an earlier indication of tumor progression or of treatment effectiveness, allow-

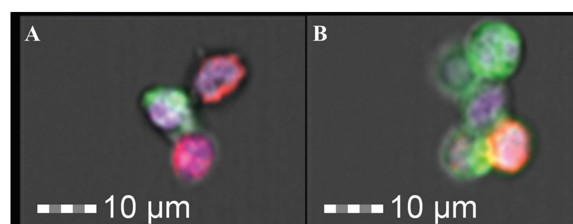

Figure 3. Imaging flow cytometry: composite images of CD45+ white blood cells (red)/CK+ (green) tumor cells (A); CK+ CTC clusters including a white blood cell, from a CRC patient's blood (B). The nuclei of the cells are stained with DAPI (purple). Images were taken at 40× magnification and have been enhanced for visual appearance.

ing chemotherapy "holidays" or a change to less aggressive therapy. Such knowledge would assist in the complex treatment decision-making process and would be welcomed by oncologists and patients alike. In conclusion, despite significant progress in our understanding of the role and value of CTCs in CRC and solid tumors generally, their clinical use as a staging tool to predict survival and response to treatment remains limited. Recent technological advances combined with a high number of large-scale clinical studies currently underway will likely fulfill the promises raised 20 years ago.

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DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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