In Response: We thank Strijbos et al. for the opportunity to further discuss our data on circulating endothelial cell (CEC) count to elucidate details that we did not comment on in our article because of space limitations.

The novel procedure described in our article enumerates a population of circulating cells with endothelial phenotype, morphology, and molecular characteristics that differ in number from the population described in previous articles from our laboratories. Transmission electron microscopy (TEM) imaging has unequivocally shown that CECs sorted according to our latest protocol are in large part nucleated necrotic cells, most likely derived from vessel wall turnover. Our novel procedure uses Syto16 staining to enumerate CECs with high DNA content. Our previous procedures, in the absence of Syto16, could not discriminate between CECs with high DNA content and macro-particles (released from CECs and circulating in the blood at numbers much higher than those of CECs with high DNA content). Nevertheless, CECs (or CEC-derived macro-particles) measured according to our previous protocols were found to have clinical prognostic and predictive potential in several clinical studies involving antiangiogenic therapies in patients with cancer (1–3). Notably, previous studies have shown that even in the absence of Syto16 staining, the cellular events counted as CD45−31°Cd146+ were not platelets because platelets did not react with the anti-CD146 antibody used in our studies (4).

Discrepancies in the number of circulating endothelial progenitors (CEP) according to different protocols are minor and reflect partially overlapping CEP phenotypes reported in the literature by different authors (e.g., CD133+ versus CD45−34+ CEPs).

In the article, we described two slightly different procedures, one, with EDTA tubes for CEC enumeration in fresh blood, and the second, with CPT tubes for CEC enumeration in frozen samples. In our experience, the use of reference fluorescent beads for enumeration in fresh blood of very rare CECs with high DNA content is technically too complex because bead enumeration requires a very low acquisition threshold associated with errantly counting cell debris in the CEC acquisition gate. Vice versa, this problem was much more relevant for CEC enumeration in frozen blood, most likely because debris were removed by mononuclear cell enrichment.

As written in the article, we acquired at least 1 × 10^6 cells in order to reach our target of 3 to 400 events in the CEC enumeration gate. This was possible in patients with a high CEC count (~1,500/mL, as stated by Strijbos et al.). In healthy subjects with fewer CECs, we acquired more cells to reach the target in the CEC acquisition gate. Had we used a reference bead enumeration strategy, several hours of acquisition time would have been required to count the requisite number of CEC events for each sample.

Finally, CECs were sorted for transmission electron microscopy from leuco-apheresis processing of several liters of blood from healthy donors or patients with cancer. In our experience, a pellet of ~500,000 cells was adequate for transmission electron microscopy analysis.

We agree with Strijbos et al. regarding the need for a consensus about CEC and CEP enumeration. However, this consensus cannot be reached in the absence of recognition of the complexity of these cell populations encompassing, among others, lymphocyte-like progenitors, necrotic mature endothelial cells, and their fragments. We hope that our experimentally validated procedure can be a step forward in this direction.

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