# Levels of Endothelial Progenitor Cells (EPCs) in smokers and moist snuff consumers

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## Abstract

Endothelial progenitor cells (EPCs) are a key cell type present in the circulation and are reported to play a role in the development of cardiovascular diseases. EPCs are derived from the bone marrow and mediate the differentiation, regeneration and maintenance of endothelial cells in response to vascular injury and angiogenesis. EPC levels have been proposed as an important biomarker for endothelial dysfunction. However, the association between levels of EPCs and endothelial dysfunction needs further investigation. Cells with the phenotype of CD34+CD133+CD309+ are defined as "triple positive" circulating EPCs. To evaluate whether EPC levels can be utilized as a smoking-related biomarker of potential harm, we measured triple positive EPC levels by flow cytometer in peripheral blood mononuclear cells (PBMCs) isolated from three different cohorts: healthy smokers (SMK) (n=40), moist snuff consumers (MSC) (n=40) and non-tobacco consumers (NTC) (n=40). We measured total EPC count, EPC percentage and EPC cumulated percentages of triple positive EPCs from the lymphocyte and monocyte populations of SMK, MSC and NTC. All the measures showed a significant increase in the EPC levels in the SMK cohort compared to NTC. The EPC levels in MSC, however, were not statistically significantly different from SMK or NTC. Higher levels of circulating EPCs in healthy smokers may be due to the increased need to repair vascular wall injury due to cigarette smoking. Further work is needed to establish the role of EPCs in understanding endothelial function and to utilize them as biomarkers of potential harm in tobacco consumers.

## Background

A key cell type in the regulation of vasculature health is the endothelial progenitor cell (EPC). EPCs are mainly derived from the bone marrow and can mediate the differentiation, regeneration and maintenance of endothelial cells in response to vascular injury or vascular angiogenesis (1,2). EPCs play a role in cardiovascular disease development, and are reported to be lower in smokers (3) and increased in those who quit smoking (4). EPCs have been proposed to be a useful biomarker for monitoring cardiovascular disease risk in those who either quit smoking or who switch to alternative tobacco products (5). EPCs are defined as triple positive, when cell surface markers are CD34+CD133+CD309+ (6,7). CD309+ cells are also known as vascular endothelial growth factor receptor 2 (VEGFR2)+ cells.

## Experimental Methods

**Study subjects and sample collection:** The blood samples for EPC enumeration were collected in a clinical study conducted by R.J. Reynolds Tobacco Company (8). Whole blood was collected from 120 generally healthy male subjects enrolled into 3 cohorts: non-Tobacco Consumers (NTC) (n = 40), smokers (SMK) (n = 40) and moist snuff consumers (MSC) (n = 40). The subjects provided written informed consent to participate in the study. The study protocol was approved by an independent Institutional Review Board and conducted per Good Clinical Practices. Processing of blood, isolation of PBMCs and other cell culture experiments were performed under sterile conditions, using microbiologically sterile supplies and reagents. PBMCs were isolated from whole blood using previously described methods (9).

**EPC identification and flow analysis:** Aliquots of cryopreserved PBMCs were thawed, washed, counted and assessed for viability. One million PBMCs were labeled with 10µl of CD34 FITC, CD133 APC, and CD309 PE antibodies for the detection of triple-positive EPCs. 7AAD was used to label the dead cells and gated on the 7AAD negative population and triple-positive EPCs. For isotype controls, mouse IgG2a FITC, mouse IgG2b APC and mouse IgG1 PE were employed. The labeling was performed for 30 min at 4˚ C. Labeled cells were washed with MACS running buffer (Miltenyi Biotech, Auburn, CA) and flow cytometry was performed. Data were analyzed using FlowJo Software (Tree Star, Ashland, OR).

**Statistical analysis:** Tukey-Kramer test of one-way mean comparisons was used to analyze the data of each endpoint. The significance of mean difference between cohorts was indicated by the Connecting Letter Report. The means of cohorts connected by same letter were not significant at 0.05 significance level. Cohorts with no connected letters were significant.

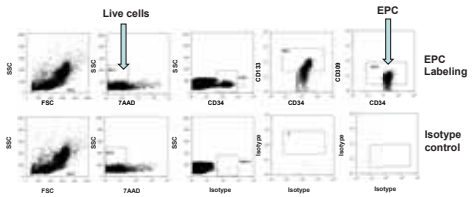
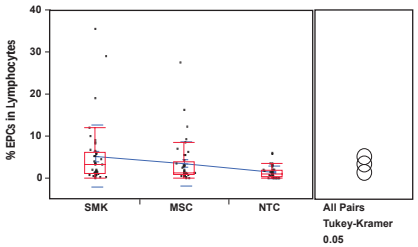
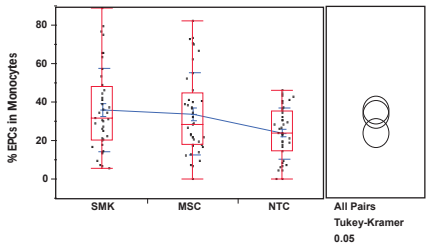



**Fig. 1. Enumeration of endothelial progenitor cells (EPCs) in PBMCs by flow cytometry:** one million PBMCs were labeled with CD34, CD133, CD309 (VEGFR2) antibodies; corresponding isotype specific antibodies were used as controls. Cells were also stained with 7AAD, a cell death marker and dead cells were excluded from the analysis. Lymphocytes and 7AAD negative cells (live cells) were gated for triple positive EPCs (top panels) and isotype controls are shown in the bottom panels.




| Cohort | Significance | Mean ± SD |
|---|---|---|
| SMK | A | 5.3±7.4 |
| MSC | A B | 3.6±5.2 |
| NTC | B | 1.5±1.5 |

**Fig. 2. Percent EPCs in Lymphocytes:** Tukey-Kramer test was used to measure the percent triple positive EPCs from different cohorts of lymphocytes. Significance and mean ± SD of different cohorts are shown in the table.




| Cohort | Significance | Mean ± SD |
|---|---|---|
| SMK | A | 36±21 |
| MSC | A B | 34±21 |
| NTC | B | 24±13 |

**Fig. 3. Percent EPCs in Monocytes:** Tukey-Kramer test was used to measure the percent triple positive EPCs from different cohorts of monocytes. Significance and mean ± SD of different cohorts are shown in the table.

## Summary and Conclusions

- A simple method to enumerate EPCs from PBMCs was developed.
- SMK have significantly higher levels of the % EPCs, cumulative % EPCs and total EPCs in lymphocytes and monocytes compared to NTC.
- Levels of the % EPCs, cumulative % EPCs and total EPCs in MSC are in between SMK and NTC, and are not significantly different from SMK or NTC. Thus, the following trend in EPC levels was observed: SMK>MSC>NTC.
- These findings differ from previous data which suggested that EPC levels are lower in smokers, relative to non-smokers (3).
- Higher levels of circulating EPCs in healthy smokers may be due to the increased need to repair vascular wall injury caused due to cigarette smoking.

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