Plasma Homocysteine, Folate, and Vitamin B₁₂ Levels in Patients With Laryngeal Cancer

Andrea Nacci, MD; Iacopo Dallan, MD; Luca Bruschini, MD; Antonio Claudio Traino, PhD; Erica Panicucci, PhD; Paolo Bruschini, MD; Valentina Mancini, MD; Ferdinando Rognini, MD; Bruno Fattori, MD

Objective: To determine plasma levels of homocysteine, folate, and vitamin B₁₂ in patients with laryngeal cancer and a control group.

Design: Analysis of homocysteine, folate, and vitamin B₁₂ levels in 25 consecutive untreated patients with laryngeal carcinoma and 80 healthy control participants. The study and control groups were subdivided into smokers, ex-smokers, and nonsmokers, as well as drinkers and nondrinkers.

Intervention: The AxSYM system was used to measure total homocysteine levels, and the ARCHITECT system (both Abbott–Diagnostics Division) was used to measure folate and vitamin B₁₂ levels.

Main Outcome Measures: Homocysteine, folate, and vitamin B₁₂ levels.

Results: The mean (SD) level of total homocysteine in patients with laryngeal carcinoma was 2.84 (1.62) mg/L vs 0.99 (0.24) mg/L in the control group (P < .001). The mean (SD) folate plasma level was 4.3 (2.2) ng/mL vs 7.9 (2.4) ng/mL (P < .001).

Conclusions: Metabolic alterations in homocysteine, folate, and vitamin B₁₂ levels, especially hypofolatemia, could be associated with laryngeal cancer. Lengthier follow-up studies and larger groups of patients will help determine the real role of these metabolic alterations.


Despite medical and surgical advances, the prognosis of patients with head and neck squamous cell carcinoma (HNSCC) has not changed substantially during the last 2 decades. In patients with early-stage tumors, second primary tumors are the most common cause of death, whereas patients with advanced head and neck tumors die mainly of locoregional disease. The development of second primary tumors is probably related to molecular abnormalities. Therefore, to elucidate the pathogenesis of HNSCC, molecular alterations have been studied extensively. Carcinogenesis is a multistep process of accumulated genetic alterations; clonal populations of cells undergo progressive genetic alterations, resulting in malignant cells with a selective growth advantage.

It is also known that carcinogenesis is associated with metabolic alterations that might promote or derive from neoplastic progression. Therefore, the study of metabolic disorders can lead to a better comprehension of tumor biological characteristics and of the tumor-patient relationship. These aspects are important in identifying new targets for therapy.

Metabolic reactions that require folate are called 1-carbon metabolism reactions. Alteration in these pathways interferes with DNA synthesis, repair, and methylation, which may promote carcinogenesis. Folate is involved in the synthesis of serine and of purine and pyrimidine bases and plays a role as methyl donor in the methionine cycle. Homocysteine is an intermediate metabolite of methionine metabolism and is metabolized in methionine or cysteine. One of these metabolic cycles leads to the production of S-adenosyl methionine, which is considered the main methyl donor in the human body.

Serum homocysteine level is a sensitive indicator of folate status. In fact, folate deficiency is often associated with hyperhomocysteinemia, and folate intake can reduce the homocysteine level. Folate deficiency aids the incorporation of uracil into the DNA, which can lead to DNA instability. High levels of homocysteine are associated with colorectal cancer, as well as uterine cervical cancer. Alterations in the methionine cycle are also thought to be involved in breast and pancreatic cancer. Although moderate folate deficiency alone might not be mutagenic in vivo, it seems to interact with other risk factors.
factors, either environmental or genetic, in promoting tumor progression.9

Elevated homocysteine plasma levels have been associated with chromosome damage even in the absence of folate or vitamin B12 deficiencies, and this finding cannot be explained by a deficient methylation of uracil to thymine. It has been shown that homocysteine can damage DNA directly via the generation of reactive oxygen species,10 and it also has been postulated that elevated plasma homocysteine levels may contribute to carcinogenesis through metal-mediated oxidative DNA damage.10 Intracellular vitamin B12 deficiency has been associated with chromosomal damage in oral mucosa cells of smokers.11 In addition, it is well known that alcohol consumption and/or cigarette smoking modify plasma levels of homocysteine, folate, and vitamin B12.12

The goal of this study was to evaluate plasma levels of homocysteine, folate, and vitamin B12 in patients with laryngeal carcinoma and to determine the role of these levels in the pathogenesis of laryngeal carcinoma.

**METHODS**

**PATIENTS**

Twenty-five consecutive untreated patients with laryngeal carcinoma were enrolled in this study (21 men and 4 women; mean [SD] age, 61.3[10.7] years; age range, 43-81 years). Informed consent was obtained from every patient. Two patients had stage I tumors; 2, stage II; 9, stage III; and 12, stage IV. Smoking habits, alcohol consumption, and presence of cardiovascular ischemic disease (CID) are outlined in Table 1.

In the study group, 11 patients had CID. 5 had hypertension (receiving pharmacological treatment), 3 had non–insulin-dependent controlled diabetes mellitus, 1 had a right bundle branch block, 1 had a mitral valve prolapse, and 1 had mild aortic valvular stenosis.

In the control group, 14 patients were being treated for hypertension, 6 had non–insulin-dependent controlled diabetes mellitus, 2 had a right bundle branch block, 2 had a mitral valve prolapse, and 1 had mild aortic valvular stenosis.

The control participants, enrolled after we obtained their informed consent, were from the same geographic areas as patients in the study group. All patients and controls followed the so-called Mediterranean diet. No patient in this study was being treated with drugs capable of altering homocysteine levels (eg, carbamazepine, phenytoin, nitrous oxide, and 6-aza-ribose triacetate). Characteristics of patients and controls are shown in Table 1.

**ANALYTICAL MEASUREMENT**

We used the AxSYM system (Abbott Diagnostics Division, Abbott Park, Illinois) for the quantitative measurement of total homocysteine in human serum. We used the ARCHITECT system (Abbott Diagnostics Division) for the quantitative determination of serum levels of folate and vitamin B12. In the study group, blood samples were drawn only at the time of diagnosis, before treatment and before patients altered their diet because of surgery.

**STATISTICAL ANALYSIS**

A t test was performed to evaluate the difference in plasma levels of homocysteine, folate, and vitamin B12 between patients and controls. A t test with Bonferroni correction was used to compare the study group with the control subgroups of smokers, ex-smokers, and nonsmokers. A t test was performed to evaluate the difference between men's and women's plasma levels of homocysteine, folate, and vitamin B12, and a correlation was made between patients' age and homocysteine, folate, and vitamin B12 levels for the study and control groups.

A stepwise regression with an outcome of laryngeal cancer was used to jointly test the effect of “predictor of pathology,” adjusting for smoking status and plasma levels of homocysteine and folate. Backward elimination was used to identify the most important prognostic factors.

All tests were considered significant at the P < .05 level. Data are given as mean (standard deviation). To convert homocysteine to micromoles per liter, multiply by 7.397; to convert folate to nanomoles per liter, multiply by 2.266; to convert vitamin B12 to picomoles per liter, multiply by 0.7378.

**RESULTS**

**HOMOCYSTEINE**

The mean level of total homocysteine among patients with laryngeal cancer was 2.83(1.62) mg/L (normal value, 0.60-0.23 mg/L) vs 0.99(0.24) mg/L in the control group (P < .001) (Figure 1). Homocysteine levels for the subgroups of both the study and control groups are given in Table 2.

A statistically significant difference in homocysteine level was seen between the 25 patients with laryngeal cancer and smoker controls (1.06 [0.30] mg/L), ex-smoker controls (1.00 [0.23] mg/L), and nonsmoker controls (0.91 [0.23] mg/L) (P < .001 for all).

In the control group, a statistically significant difference in homocysteine level was observed between smokers and nonsmokers (P = .04).

Neither alcohol intake nor presence of cardiovascular disease altered the statistically significant difference

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**Table 1. Study and Control Group Variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients With Laryngeal Cancer (n=25)</th>
<th>Control Group (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>61.3 (10.7)</td>
<td>56.0 (15.1)</td>
</tr>
<tr>
<td>Male sex, No. of participants</td>
<td>21</td>
<td>43</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokersa</td>
<td>13 (62)</td>
<td>25 (31)</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>0</td>
<td>30 (38)</td>
</tr>
<tr>
<td>Ex-smokersb</td>
<td>12 (48)</td>
<td>25 (31)</td>
</tr>
<tr>
<td>CID status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CID</td>
<td>11 (44)</td>
<td>26 (33)</td>
</tr>
<tr>
<td>No CID</td>
<td>14 (56)</td>
<td>54 (68)</td>
</tr>
<tr>
<td>Drinking alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drinkersc</td>
<td>16 (64)</td>
<td>48 (60)</td>
</tr>
<tr>
<td>Nondrinkers</td>
<td>9 (36)</td>
<td>32 (40)</td>
</tr>
</tbody>
</table>

Abbreviation: CID, cardiovascular ischemic disease.

a Data are given as the number (percentage) of participants, unless otherwise indicated.

b Smoked 20 cigarettes or more per day.

c Had smoked 20 cigarettes or more per day for at least 1 year.

d Drank 500 mL or more of wine per day or 1000 mL or more of beer per day (dosage was halved for women).
in homocysteine level between patients with laryngeal carcinoma and the control group.

In the study group, no statistically significant difference in homocysteine level was noted between drinkers and nondrinkers or between patients with CID and those without CID. However, in the control group, a statistically significant difference was found between drinkers and nondrinkers (mean [SD], 0.91 [0.23] mg/L vs 1.04 [0.26] mg/L; \( P = .01 \)) and between participants with and without CID (1.12 [0.24] mg/L vs 0.93 [0.23] mg/L; \( P = .003 \)).

No correlation between age and homocysteine level was found in the study group (\( R = -0.23 \)) or in the control group (\( R = 0.23 \)). In addition, there was no statistically significant difference in plasma homocysteine level between men and women, and there was no correlation between homocysteine level and tumor stage.

**FOLATE**

The mean plasma folate level among patients with laryngeal cancer was 4.3 [2.2] ng/mL (normal value, 2.7-34.0 ng/mL) vs 7.9 [2.4] ng/mL in the control group (\( P < .001 \)) (Figure 2).

The mean folate level in the study and control subgroups are given in Table 2.

A statistically significant difference was observed between all patients and each of the 3 control subgroups (smokers, 7.5 [1.9] ng/mL; ex-smokers, 8.0 [2.5] ng/mL; nonsmokers, 8.1 [2.8] SD) (\( P < .001 \) for all). No statistically significant difference in folate plasma levels was detected among the 3 control subgroups.

Neither alcohol intake nor cardiovascular disease altered the statistically significant difference found in folate levels between the study and control groups.

In the study group, there was no statistically significant difference in folate levels between drinkers and nondrinkers or patients with and without CID. Similarly, in the control group, no statistically significant difference in folate levels was seen either between drinkers and nondrinkers or between participants with and without CID.

No correlation between age and folate plasma level was found either in the study group (\( R = -0.22 \)) or the control group (\( R = -0.03 \)).

No statistically significant difference in plasma levels was noted between men and women, and no correlation was found between folate level and tumor stage.

### Table 2. Homocysteine, Folate, and Vitamin B12 Levels in the Study and Control Groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Smokers</th>
<th>Ex-Smokers</th>
<th>Smokers</th>
<th>Ex-Smokers</th>
<th>Smokers</th>
<th>Ex-Smokers</th>
</tr>
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<tr>
<td>Patients With</td>
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<td></td>
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</tr>
<tr>
<td>Laryngeal Cancer</td>
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<td></td>
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<tr>
<td>Control Group</td>
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<td></td>
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</tbody>
</table>

SI conversion factors: to convert homocysteine to micromoles per liter, multiply by 7.397; folate to nanomoles per liter, multiply by 2.266; vitamin B12 to picomoles per liter, multiply by 0.7378.

\( a \) Data are given as the number (percentage) of participants, unless otherwise indicated.

\( b \) There were no nonsmokers in the study group.

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Figure 1. Plasma homocysteine levels and coefficient variation percentage for patients with laryngeal cancer and controls. Lines represent mean levels of homocysteine in the study group (dashed) and controls (solid).

Figure 2. Plasma homocysteine levels and coefficient variation percentage for patients with laryngeal cancer and controls. Lines represent mean levels of homocysteine in the study group (dashed) and controls (solid).
VITAMIN B₁₂

The mean plasma level of vitamin B₁₂ was 385 (278) pg/mL in patients (normal value, 179-1162 pg/mL) vs 498 (125) pg/mL in the control group (P < .01). Vitamin B₁₂ levels for the study and control subgroups are summarized in Table 2. Neither alcohol intake nor cardiovascular disease affected vitamin B₁₂ levels. No correlation was found between age and vitamin B₁₂ level for the study group (R = 0.05) or the control group (R = −0.29). No statistically significant difference in vitamin B₁₂ level was noted between men and women, and no correlation was found between vitamin B₁₂ level and tumor stage.

MULTIVARIATE ANALYSIS

The model for the multivariate analysis took into account smoking status and plasma levels of homocysteine and folate because of the statistical significance of these variables in a univariate analysis, which excluded other factors. Stepwise regression analysis showed that the strongest independent risk factor for laryngeal cancer was homocysteine level (P < .01; F, 9.56) followed by folate level (P < .01) (Table 3).

COMMENT

The relationship among homocysteine levels, folate levels, and HNSCC is still a matter of discussion. Almadori et al. found a significant correlation between low serum folate levels and high serum homocysteine levels in patients with HNSCC. In this study, we found a statistically significant difference in homocysteine and folate plasma levels between the laryngeal cancer and control groups. In addition, there was a statistically significant difference in vitamin B₁₂ levels between the study and control groups. These findings are not surprising, given the fact that folate and vitamin B₁₂ are correlated in the methionine cycle.

In this study, we focused our attention on the methionine cycle, which is very important in cellular biochemistry because it leads to the production of the universal methyl donor, S-adenosyl-methionine. Alterations in the methionine cycle have been described in many human malignant neoplasms. Moreover, genetic alterations in the enzymes involved in the methionine cycle have been described recently in patients with head and neck cancer.

The role of these alterations in neoplastic progression is far from clear. At this point, it is essential to understand whether these alterations are markers of risk or a consequence of the HNSCC. Low plasma folate levels have been considered a risk factor for head and neck multistep carcinogenesis, even though hypofolatemia is not considered to have an independent role as the initiating factor. Nevertheless, if these conditions precede the development of HNSCC it should be possible to identify a group of subjects with increased risk; with this in mind, prophylaxis by means of folate therapy could be administered in these cases. In fact, it is well known that an increase in folate intake is associated with a decrease in the risk of oral cancer, even if this relationship is just owing to folate intake from fruit.

Currently, folate is thought to be protective against colorectal carcinoma, and higher serum homocysteine levels and lower serum folate levels are seen in patients with this neoplasm. Another report seems to confirm the protective role of folate against human malignant neoplasms, and alterations in these metabolic pathways also have been observed in patients with breast and pancreatic cancer. Until now, serum homocysteine and folate levels have not been considered markers of risk of cervical dysplasia, although other authors report different conclusions regarding the role of hyperhomocysteinemia as a marker of cervical cancer risk.

From a biomolecular point of view, hyperhomocysteinemia is considered an important risk factor for ge-
nomic instability. Folate deficiency can lead to inappropriate activation of proto-oncogenes and repression of tumor suppression genes by altering cytosine methylation in DNA. Folate is also fundamental in normal DNA synthesis and repair; moreover, normal levels of precursor nucleotides (deoxyribonucleotide triphosphate) are directly dependent on intracellular folate availability, and the carcinogenic effect of folate deficiency seems to be based, in part, on folate pool imbalance.

Homocysteine can damage DNA by altering thymine and guanine residues in the presence of Cu$^{2+}$, even in the presence of a normal folate and vitamin B12 concentration. Alterations in DNA methylation, especially in promoters of tumor-involved genes, are common in patients with HNSCC.

Cobalamin, or vitamin B12, is a coenzyme of methylmalonic coenzyme A mutase and of methionine synthetase, and it is fully involved in the methionine cycle. Moreover, folate and vitamin B12 supplementation have been demonstrated to induce regression of bronchial squamous metaplasia.

In a comparison of patients with laryngeal cancer with a control group, we noted statistically higher levels of homocysteine and lower levels of folate among patients ($P < .001$). Moreover, in the study group, the plasma levels of these proteins had no connection with alcohol consumption, smoking habits, or the presence of CID. This apparently incongruent situation might derive from the association with the tumor or from the small study population and/or inadequate statistical power.

It is possible to consider individuals at risk, such as men who drink more than 500 mL of wine a day or more than 1000 mL of beer a day (for women, that dosage is halved). There are many reports in the literature suggesting that the consumption of great quantities of alcohol ($\geq 500$ mL of wine a day or $\geq 1000$ mL of beer a day) and that cigarette smoking ($\geq 20$ cigarettes per day) can alter the metabolic pathway of homocysteine and folic acid. In particular, alcohol is capable of interacting with the methionine cycle through an increase in methionine adenosyltransferase, variations in S-adenosylmethionine and S-adenosylhomocysteine levels, a reduction in methionine synthase, and an increase in 5-methyl-tetrahydrofolate and betaine-homocysteine methyltransferase. A modification in the methionine cycle eventually results in an increase in homocysteine levels in the blood.

Various authors report that cigarette smoking also influences the levels of homocysteine, folate, and vitamin B12 in the blood because of the alterations that take place in the methionine cycle and the fact that smokers generally eat fewer fruits and vegetables, the main source of folate and vitamin B12.

In the group of patients that we studied, we saw high levels of homocysteine and low levels of folate in both the subgroup of drinkers and the nondrinking group; the same results were seen for smokers and ex-smokers. These data, as demonstrated also by the stepwise regression analysis, indicate that hyperhomocysteinemia and hypofolatemia are connected more to the presence of the tumor than to alcohol consumption or cigarette smoking. Confirmation is seen in the fact that, in the control group, drinkers had significantly higher levels of homocysteine than nondrinkers ($P = .01$), as did smokers compared with nonsmokers ($P = .04$).

The association between high homocysteine levels and cardiovascular disease is well known. In our study group, however, high homocysteine levels were independent of CID, whereas controls with CID had significantly higher homocysteine levels than controls without CID ($P = .003$). These data further corroborate the hypothesis that high homocysteine levels in patients with laryngeal cancer are associated with the presence of the tumor rather than CID.

In patients with laryngeal cancer, the plasma homocysteine levels were very heterogeneous, as demonstrated by the high standard deviation (1.62 mg/L). Perhaps homocysteine levels are influenced not only by plasma folate levels but also by tumor phenotype. Therefore, higher levels of methionine can be caused by a genetic alteration in the methionine cycle. This condition has been described recently in ovarian neoplastic cells, and, theoretically, drugs can target this metabolic alteration.

### Table 3. Stepwise Multiple Regression

<table>
<thead>
<tr>
<th>Variables in Multiple Regression Analysis Model</th>
<th>Coefficient</th>
<th>SE</th>
<th>Standard Coefficient</th>
<th>F-to-Remove</th>
<th>$R^2$</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.110</td>
<td>0.120</td>
<td>0.110</td>
<td>0.836</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td>0.071</td>
<td>0.036</td>
<td>0.133</td>
<td>3.852</td>
<td>0.558</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Homocysteine level</td>
<td>0.029</td>
<td>0.004</td>
<td>0.553</td>
<td>51.559</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate level</td>
<td>-0.037</td>
<td>0.012</td>
<td>-0.242</td>
<td>9.929</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.194</td>
<td>0.114</td>
<td>0.194</td>
<td>2.912</td>
<td>0.542</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Homocysteine level</td>
<td>0.030</td>
<td>0.004</td>
<td>0.571</td>
<td>54.477</td>
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<tr>
<td>Folate level</td>
<td>-0.039</td>
<td>0.012</td>
<td>-0.259</td>
<td>11.213</td>
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<tr>
<td>Step 1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.152</td>
<td>0.049</td>
<td>-0.152</td>
<td>9.558</td>
<td>0.491</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Homocysteine level</td>
<td>0.037</td>
<td>0.004</td>
<td>0.701</td>
<td>99.432</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$a$ Dependent variable was the presence or absence of laryngeal cancer; independent variables were smoking status and plasma homocysteine and folate levels.
Plasma folate and homocysteine levels were seen to not vary with the different tumor stages, and, therefore, they cannot be considered as a marker of disease progression. Moreover, plasma folate levels in patients with HNSCC are similar to those found in patients with leukoplakia and, for this reason, cannot be considered as a diagnostic marker of HNSCC. However, keeping in mind the function of folate in DNA synthesis and repair, a speculative role in carcinogenesis can be hypothesized. Plasma folate level probably does not play an independent role as an initiating factor but rather interacts with either environmental or genetic factors.

In addition, folate intake within the current recommended range is often insufficient for achieving the optimal concentration of precursor nucleotides in the cells, and an increase in the recommended dietary allowances for folate and other micronutrients has been proposed. Second primary tumors are the main cause of death in patients with early-stage HNSCC, whereas locoregional failure is the main cause of death in patients with advanced-stage HNSCC. Based on these data and assuming that folate deficiency and hyperhomocysteinemia can predispose patients to develop head and neck cancer, it is logical to consider using folate as a chemopreventive agent in patients with HNSCC, as in colon cancer treatment. Moreover, folate supplementation could prove a simple and inexpensive preventive measure.

In conclusion, we found an interesting association between plasma homocysteine and folate levels and laryngeal cancer. There are some cellular biochemistry theories that might support this association between altered methionine cycle and DNA synthesis and repair, nevertheless, a longitudinal study of larger groups of patients with precancerous lesions is necessary, as are lengthier follow-ups, to define the real role of these metabolic alterations.

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Correspondence: Andrea Nacci, MD, Third Otorhinolaryngology Unit, Department of Neurosciences, University of Pisa, Via Savi No. 10, 56126 Pisa, Italy (a.nacci@med.unipi.it).

Author Contributions: Drs Nacci, Dallan, and Fattori had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Nacci and Fattori. Acquisition of data: Dallan, L. Bruschini, and Mancini. Analysis and interpretation of data: Nacci, Traino, Panicucci, P. Bruschini, and Rognini. Drafting of the manuscript: Dallan, L. Bruschini, Traino, P. Bruschini, Mancini, and Rognini. Critical revision of the manuscript for important intellectual content: Nacci, Panicucci, and Fattori. Statistical analysis: Traino, Panicucci, and Mancini. Obtained funding: P. Bruschini. Administrative, technical, and material support: Dallan and L. Bruschini. Study supervision: Nacci and Fattori.

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