RANK-L is a Potential Therapeutic Target in Homogeneous Atherosclerotic Plaques

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Abstract

Objective and design: The late stages of carotid atherosclerosis are responsible for increased local stiffness, suggesting the need for a therapeutic target that affect either plaque composition and arterial stiffness. There is a lack of data on the role of the local arterial stiffness, assessed by radio-frequency based system and its relationship with the molecular profile of plaques.

Subjects: In this study we enrolled 18 consecutive patients undergoing carotid endarterectomy, with homogeneous or heterogeneous plaques, as established by Doppler-ultrasound and local pulse-wave velocity was assessed before surgery.

Methods: In carotid plaque specimens, we evaluated inflammasome (NLRP3), Receptor Activator of Nuclear Factor κB (RANK) and its natural ligand (RANK-L), Osteoprotegerin (OPG), and other inflammatory and apoptotic molecules by Western Blotting and qPCR analysis. In addition, lipid peroxidation of arterial specimens was assessed by TBARS assay.

Results: In heterogeneous plaques we observed increased OPG expression (p=0.04), positively correlated with lipid peroxidation values (r=0.511, p=0.03); increased levels of RANK (p=0.02), and other inflammatory and apoptotic molecules. RANK-L protein was augmented in homogeneous plaques (p=0.01) and correlated to ß-index (r=0.514, p=0.03) and PWV (r=0.525, p=0.03) values.

Conclusions: Our data provide evidence that increased local PWV and ß-index might identify plaque evolution towards calcification.

Keywords: Carotid plaque; Inflammasome; Stiffness; RANK-L; OPG

Introduction

Atherosclerosis, causing the most of cerebrovascular disease, represents the prevalent cause of death and disability in the Western countries. Atheroma is the characterizing lesion of the inner layer of large and medium size arteries, it develops over decades, during which remains clinically silent. When a plaque become bulky, several complications may arise: the inner layer could tears allowing clot formation, or the plaque structure could switch from fibrous to necrotic lesion. In this latter case, plaque rupture can occurs with consequent presence of liquefied tissues and remnants of necrosis that could migrate to the brain, causing embolism. A plaque with a large lipid pool, a thin cap and macrophage-dense inflammation on or beneath its surface is named vulnerable plaque, otherwise susceptible to injury or susceptible to attack [1]. A crucial moment in atherogenesis is established when phagocytes lose their function and become unable to remove apoptotic cells from the environment, and as a result, a reduced uptake of apoptotic cells promotes plaque instability and necrosis [2]. Carotid plaques that contain a large necrotic core, intraplaque haemorrhage, or thin fibrous cap, so-called vulnerable plaques, have been described to increase the risk of cardiovascular events [3]. Calcification, on the other hand, is another common complication of the atherosclerotic plaques and it is associated with lipid-laden and flow-limiting in correspondence to the atherosclerotic lesion. It is well known that later stages of plaque evolution to calcification are influenced by the OPG/RANK-L pathway [4], and that the expression of RANK-L can be increased by inflammatory cytokines, such as TNFα and IL-1β [5]. All these pathophysiological evidences underline the importance to examine factors that may lead to atherosclerosis and particularly affect plaque evolution. Arterial stiffness plays a crucial role as trigger of atherosclerotic process and for maintaining its progression, as well. Moreover, an increased aortic stiffness was shown as an independent risk factor for stroke and cardiovascular mortality [6]. Previous studies have investigated the relationship between arterial stiffness and aortic or coronary calcifications [7]. However, the relationship between arterial stiffness and carotid plaque components such as inflammation, apoptosis, haemorrhage etc., has received less attention. Although arterial stiffness and atherosclerosis share some common determinants, such as increasing age, sex, and hypertension, little is known whether an
Materials and Methods

Study subjects, clinical assessment and surgery

In this study, 18 consecutive patients (14 males, mean age 72.3 ± 8.4 years; 4 females, mean age 73.5 ± 7.04 years) undergoing carotid endarterectomy in our University Hospital between January and June 2013, were enrolled. Indications for surgery were: asymptomatic carotid stenosis with lumen obstruction >75%; or symptomatic carotid stenosis with a lumen obstruction >70%. Evaluation of carotid plaques was performed before surgery by echo Doppler, through the My-Lab 70 ultrasound system (Esaote, Florence, Italy) equipped with a linear probe with a frequency of 10 MHz. Based on plaque's composition, the lesions were classified as heterogeneous and homogeneous, according to Bluth's classification [14]. Accordingly, patients were divided into two groups: those with heterogeneous and those with homogeneous plaques. As parameters of local arterial stiffness, PWV and β-stiffness index were recorded. The surgery was performed in a standard fashion, with an oblique transection of the carotid artery, followed by an eversion of the vessel wall to access and remove the plaque. A sample of carotid plaque tissue was immediately stored at -20°C and later used for Western Blotting, Real-Time PCR and TBARS analyses.

Study of arterial stiffness

Measurements of local arterial stiffness were obtained at the level of the common carotid arteries at about 2 cm proximal to the bifurcation, in order to avoid any influence of the complex flow in the carotid sinus. A My-lab 70 ultrasound system (Esaote, Florence, Italy) equipped with a high-definition echo-tracking package (Quality Arterial Stiffness) was employed. For the evaluation, subjects lay down in the supine position and rested for 10-15 min. Brachial blood pressure (BP) measurements were performed, just before starting of the carotid study, by a single investigator (MM) in a quiet room with the subjects at a supine position and with the arterial wall visible throughout the entire screen during the scanning. The movement of carotid walls is tracked in the entire region of interest (green rectangle width 15 mm); Continuous red lines indicate the automatic positioning of wall-tracking points at media–adventitia interface. Continuous green lines display dynamically the amplified vessel wall movement; Real-time distension waveforms are displayed at the bottom (blue line). The values of carotid distension (DIST) and minimum diameter (D) are displayed beat-to-beat on the screen, and the mean value (MED) over the last six beats and SD and SD are continuously calculated; Standard deviation (SD) value, displayed on the side of the region of acquisition (ROI), provides the user a real-time quality feedback about data acquisition. This value will be orange when SD value is >21 and green when it is <21, as in this case; the more homogeneous the measure is, the more accurate the measure of local stiffness will be. Quality AS automatically calculated the modification of the arterial diameter between the systolic and diastolic phases. Theoretically, carotid diameter waveforms were assessed by means of ultrasound and converted to carotid pressure waveforms using an empirically derived exponential relationship between pressure and arterial cross-section. The derived carotid pressure waveform is calibrated to brachial end diastolic and mean arterial pressure by iteratively changing the wall rigidity coefficient. This allows the calculation of the arterial stiffness [15]. Carotid stiffness indices: pulse wave velocity (PWV, m/s), distensibility coefficient (DC, 1/KPa), compliance coefficient (CC, mm²/kPa), α, β and augmentation index (AIx, %) were obtained. For this study, only PWV and Stiffness index β were evaluated.

Specifically, PWV was calculated from the following equation:

\[
PWV=\sqrt{\frac{1}{\rho} \times DC=\sqrt{D_2 \times \Delta p/p \times (2 \times D \times \Delta D_2)}
\]

Where D: Diastolic diameter; \(\Delta D\): Change of diameter in systole; DC: Distensibility coefficient; \(\Delta p\): Local pulse pressure; \(p\): Blood density (\(p = 1.050 \text{ kg/m}^3\)).

Stiffness index β was expressed as:

\[
\text{ln}(SP/DP) \times D/\Delta D
\]
where SP and DP are carotid systolic and diastolic pressure respectively.

For establishing the functional status of arterial wall in our cohort we compared stiffness parameters (PWV and Stiffness index β) to those from 45 age- and gender-matched asymptomatic subjects undergoing echo Doppler examination for screening of atherosclerosis, as the example reported in Figure 1.

Physicians (CZ and GA) that performed echo Doppler examinations were blinded from patient clinical history.

### Western blotting analysis

Samples from carotid plaques (50 mg) were homogenized in lysis buffer (1% Triton; 20 mMTris/HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 5 mM EDTA; 1mM phenylmethylsulfonyl fluoride; 1% aprotinin; 15 µg/mL leupeptin), centrifuged at 15000 rpm for 15 min at 4°C and the protein content was determined by using the DC protein assay (Biorad, Milan, Italy); OPG and RANK (Chemicon, Temecula, CA); RANK-L, HMGB1 and ASK1 (Abcam ltd., Oxford, UK); p-ERK, p-JNK (Cell Signalling, Beverly, MA).

The protein sample was quantified by scanning densitometry using a bio-image analysis system ECL plus (Thermo Scientific, Waltham, MA). The results were expressed as relative integrated intensity subtracting the respective backgrounds. More in detail the background was represented by β-actin expression that is used to normalize the results of densitometry analysis.

### TBARS assay

Samples were homogenized in 1,15% KCl (1 mL/250 mg), centrifuged for 20 min at 4000 rpm and 100 mL of supernatant were added in tubes containing 10 mL of a solution composed of: SDS 8.1%, acid acetic solution 20%, TBA solution 0.8% and distilled water. Standard curve (1,1,3,3-Tetramethoxyxpropane), samples and blank control were boiled at 95°C for 1 h, then loaded twice into a plate and absorbance was read at 540 nm.

### Real time PCR analysis

Tissue samples (50 mg) were homogenized in QIAzol Lyses Buffer (1 mL) for RNA extraction according to the manufacturer’s protocol (Qiagen, Venlo, Limburg, Netherlands). After reverse transcription, trough cDNA Archive High-Capacity Reverse Transcription kit (Life Technologies, Foster City, CA), the cDNA was stored at −80°C and was used to quantify by TaqMan® Gene Expression Assay the amount of Ras (Hs00163653_m1), Ras-L (Hs00181225_m1), Bcl-2 (Hs01923466_g1), β-actin (Hs02742609_g1) cDNA as endogenous control. The reaction was performed on the SDS 7300 instrument from Life Technologies and the relative expression was quantified by the with 2-ΔΔCt method. Sample with higher ΔCt was used as arbitrary calibrator.

### Statistical analysis

All data are expressed as median and IQR. Comparisons between variables were assessed by Mann-Whitney t-tests, and correlations were evaluated by Spearman’s correlation. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Graphs were drawn using Graph Pad Prism (version 5.0 for Windows). Of the analysed subjects, 4 (two for each group) had not detectable levels of expression of most of the examined molecules and were excluded from statistical analysis.

### Results

#### Characteristics of the study subjects

Demographic and clinical characteristics of patients with homogeneous plaque and those with heterogeneous lesion are detailed in Table 1. Local stiffness values and clinical parameters between patients were compared with Mann-Whitney t-test. There was no difference with regard to demographic and clinical (blood pressure values, risk factors, therapy) variables between the two groups of patients. Before surgery, all patients with heterogeneous plaque experienced brain-vascular events and showed at ultrasonographic examination a plaque determining a stenosis >70%, whereas patients with homogeneous plaque, were asymptomatic and showed a plaque with a stenosis >75%.

Local stiffness was significantly increased in all patients compared to controls (PWV=10.6 ± 2.1 vs. 7.3 ± 1.3 m/sec, p<0.001; β-index=18.56 ± 6.2 vs. 9.8 ± 4.88, p<0.001). The β-index results did not show statistical differences between groups.

### Table 1: Demographic and clinical characteristics of patients with homogeneous plaque and those with heterogeneous plaque

<table>
<thead>
<tr>
<th>Demographic and clinical features</th>
<th>Homogeneous (n=7)</th>
<th>Heterogeneous (n=6)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years ± SD</td>
<td>70.85 ± 8.11</td>
<td>74.83 ± 9.10</td>
<td>0.352*</td>
</tr>
<tr>
<td>Male gender, n(%)</td>
<td>6 (86)</td>
<td>3 (50)</td>
<td>0.212*</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg ± SD</td>
<td>140.21 ± 20.90</td>
<td>136.66 ± 8.16</td>
<td>0.806*</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg ± SD</td>
<td>75.71 ± 10.17</td>
<td>76.66 ± 5.16</td>
<td>0.601*</td>
</tr>
<tr>
<td>Pulse pressure, mmHg ± SD</td>
<td>65 ± 13.54</td>
<td>60 ± 8.94</td>
<td>0.706*</td>
</tr>
<tr>
<td>Hypertension, n(%)</td>
<td>6 (86)</td>
<td>5 (83)</td>
<td>1*</td>
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<tr>
<td>Diabetes, n(%)</td>
<td>3 (43)</td>
<td>1 (17)</td>
<td>0.373*</td>
</tr>
<tr>
<td>Smoking, n(%)</td>
<td>3 (43)</td>
<td>3 (50)</td>
<td>0.869*</td>
</tr>
<tr>
<td>Statin, n(%)</td>
<td>7 (100)</td>
<td>1 (17)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Pulse Wave Velocity, m/s ± SD</td>
<td>10.79 ± 2.50</td>
<td>11.42 ± 0.83</td>
<td>0.234*</td>
</tr>
<tr>
<td>β-index</td>
<td>18.56 ± 4.33</td>
<td>21.68 ± 3.35</td>
<td>0.366*</td>
</tr>
</tbody>
</table>

*Two-tailed p-value using Mann-Whitney test

Lipid peroxidation quantification

Oxidative stress in arterial specimens was estimated by TBARS assay evaluating by spectrophotometric measurements the main products of lipid peroxidation, namely Malondialdehyde (MDA) and 4-Hydroxynonenal (HNE). Values were compared by Mann-Whitney test demonstrating an increased presence of lipid peroxidation in heterogeneous plaques (p=0.02, Figure 2).

**Figure 2:** The graph represents lipid peroxidation values in the two groups, Mann-Whitney U-test (one-tailed) showed increased TBARS values in heterogeneous plaques (p=0.02).

Protein expression profile of carotid plaques

In this study we examined the NLRP3 inflammasome activation and the MAPK cascade, which identify p-ERK and p-JNK as the main effectors. The expression of NLRP3 in plaque specimens did not differ between groups (results not shown, p=0.44). Despite the difference was not significant, the heterogeneous plaques showed an increased presence of this protein, probably as a consequence of the higher levels of lipids peroxidation in these plaques.

The two MAPKs, evaluated in their phosphorylated forms, resulted more activated in heterogeneous plaques. Specifically p-ERK 1/2 expression demonstrated a (p=0.03; Figure 3A) and p-JNK 1/2/3 levels were significantly higher in heterogeneous plaques (p<0.03; Figure 3B).

Deregulation of apoptotic pathway in atherogenesis is well known with the oxidant status leading to increasing pro-inflammatory cytokines production, thus we investigated two early and delayed apoptosis markers, namely ASK1 and HMGB-1. The endogenous levels of ASK1 were higher in heterogeneous than homogenous (p=0.01; Figure 3C), while HMGB-1 protein did not show significant differences between the two groups (not shown).

Plaque stability is often associated with its phosphorylation which reduces ruptures and for this reason we investigated RANK-L/OPG/RANK axis that has pleiotropic effects on inflammation. In heterogeneous plaques we observed higher levels of both RANK (p=0.03; Figure 4A) and OPG (p=0.04; Figure 4B) expression. On the contrary, homogeneous plaques showed a higher expression of RANK-L protein (p=0.01; Figure 4C).

**Figure 3:** The graph in A represents p-ERK 1/2 western blotting results in the two groups. Mann-Whitney U t-test showed increased levels in heterogeneous plaques (**p=0.03); The graph in B represents p-JNK 1/2/3 western blotting results, showing increased levels in heterogeneous plaques (**p<0.03); The graph in C represents ASK1 western blotting results indicating increased in expression heterogeneous than homogenous plaques (**p=0.01); The representative blots, for each group, were selected and cropped from the same x-ray film; The same membrane was used to evaluate p-ERK, p-JNK, and β-actin, thus the same band of β-actin is represented in A and B.

**Figure 4:** The graphs in A, B, and C represent, respectively, western blotting results of RANK, RANK-L and OPG; Mann-Whitney U t-test showed higher levels of RANK and OPG (§p=0.02 and *p=0.04 respectively) proteins in heterogeneous plaques; RANK-L western blotting results, showing higher levels in homogenous plaques (*p=0.01); The representative blots, for each group, were selected and cropped from the same x-ray film.

Plaque composition differentially impact on mRNA profile

We investigated both inflammatory and apoptotic molecules. Regarding apoptosis, the extrinsic pathway was evaluated comparing FAS vs. FAS-L expression levels, these showed an increased expression in heterogeneous plaques compared to homogenous (p=0.019; Figure 5A). This could reflect, in part, the less susceptibility to rupture of homogeneous plaques. As a compensatory mechanism the analysis of...
Bcl2 revealed an increased expression in heterogeneous plaques compared to homogeneous (p<0.0001; Figure 5B). The intrinsic pathway evaluated by caspase-8 mRNA expression did not reflect any change between groups (data not shown).

As a further apoptotic mechanism we compared the expression of TNFα and its specific receptor TNFR-II observing an increased expression of these mRNAs in heterogeneous plaques (p<0.0001; Figure 5C). Since the activation of TNFRII determines the release and further production of HMGB-1, we compared the expression of these two mRNAs evidencing a higher expression in heterogeneous plaques (p=0.04; Figure 5D), suggesting a boosted activation of the TNF pathway in this kind of plaques presenting a necrotic core and causing cerebrovascular events.

![Figure 5: Graphs represent Real Time PCR results; Mann-Whitney U t-test showed higher levels of both A FAS/FAS-L (***p=0.019), B Bcl-2 (**p=0.0001), C TNFα/TNFR-II ratio (**p=0.0001) and D HMGB-1/TNFR-II ratio (*p=0.04) in heterogeneous plaques.](image)

Correlation analysis

To evaluate if a correlation could exist between molecular parameters and parameters of local functional arterial properties, we performed further analysis, summarized in Tables 2 and 3. Of the several analysed parameters, there was a positive correlation between TBARS levels and OPG values (r=0.511, p=0.03; Table 2). Moreover, β-index and PWV showed a positive correlation with RANK-L levels (r=0.514, p=0.02; and r=0.525, p=0.03 respectively) as reported in Table 3. None of the other evaluated molecules demonstrated significant correlations with either stiffness parameters or lipid peroxidation (Tables 2 and 3).

![Table 2: Correlation analysis between TBARS levels and western blotting results of the analyzed proteins; A positive correlation was found between TBARS levels and OPG values (r=0.511, p=0.03).](table)

![Table 3: Correlation between parameters of local functional arterial properties and western blotting results of the analyzed proteins; β-index and PWV showed a positive correlation with RANK-L levels (r=0.525, p=0.03; and r=0.514, p=0.03 respectively).](table)

Discussion

In this prospective study, a small sample of patients with advanced atherosclerosis were tested for arterial stiffness and its association with inflammatory and apoptotic markers. The main finding of our research suggests that RANK-L is significantly associated with plaque calcification and increased values of local PWV and β-index.

Arterial stiffness is the principal cause of increasing systolic blood pressure and pulsatility of flow, and therefore, PWV may reflect the cumulative damage on the arterial wall. An independent predictive value of arterial stiffness for predicting cerebrovascular events has been reported in the literature [6,16,17]. Therefore, there is an increasing interest for an extensive evaluation of these parameters, particularly in patients at major risk. Recently, new radio frequency based software for assessing local arterial stiffness, and therefore, PWV may reflect the cumulative damage on the arterial wall. An independent predictive value of arterial stiffness for predicting cerebrovascular events has been reported in the literature [6,16,17]. Therefore, there is an increasing interest for an extensive evaluation of these parameters, particularly in patients at major risk. Recently, new radio frequency based software has been introduced, as alternative approach over conventional method, for assessing local arterial stiffness in clinical routine [9-13]. However, in our knowledge, studies investigating the relation between arterial stiffness, by echo-tracking systems, and plaque composition are lacking to date. In the present study, symptomatic and asymptomatic subjects, with different plaque morphology were involved. Although a significant increase in arterial stiffness was well detected in our
patients compared to healthy controls, we could not confirm that local PWV is a useful parameter to evaluate the relative risk of CVE in subjects with heterogeneous (vulnerable) plaques. This is owing to the lack of significance in PWV differences between subjects with heterogeneous or homogeneous lesions. One explanation for this result, partially dependent on the small number of patients recruited, may be that PWV by echo tracking better reflects vessel stiffness on the calcified site, being a local measurement of functional artery properties. This was confirmed by the evidence of a significant correlation between RANK-L, more expressed in homogeneous (calcified) plaques, and either β-index and PWV, suggesting that local arterial wall characteristics might affect plaque calcification. Nevertheless, in a recent large population-based cohort from the Rotterdam Study, a higher PWV was associated with presence and composition of carotid atherosclerotic plaques, in particular with intraplaque hemorrhage, whereas associations between arterial stiffness and lipid or calcification were less pronounced [18]. However, a direct comparison between our results and those from the Rotterdam Study is not possible for several reasons: larger number of recruited patients, use of magnetic resonance imaging (MRI) for the assessment of plaque composition and conventional measurement of PWV by two-points method. Moreover, whether findings from the Rotterdam provide essential clues for understanding the development of vulnerable atherosclerotic plaque, a major advantage of our research is that we examined molecular expression profile directly on carotid plaque specimens, differently from the Rotterdam cohort in which plaque components (lipid, calcification, hemorrhage) were indirectly assessed through MRI.

The presence of RANK-L in atherosclerotic lesions denotes their development towards calcification, partly due to the old age, to the recruitment of circulating Ca2+ by Vascular Smooth Muscle Cells (VSMC), and also by a de-regulation of the RANK/RANKL/OPG pathway. Recently, an increasing number of animal and human studies have been performed about the potential role of OPG/RANKL in the development of vascular disease [19,20]. OPG and RANKL elevated levels are associated with the presence, severity and progression of cardiovascular disease, including carotid atherosclerosis [21,22]. Elevated OPG levels have been associated with the progression of vascular calcification in patients receiving long-term hemodialysis; OPG levels can explain for the association between coronary artery calcification and chronic kidney disease [23]. OPG represents a novel marker of cardiovascular mortality and clinical events in patients with acute myocardial infarction complicated with heart failure [24]. However, there has been little clinical evaluation about the association between circulating OPG and RANK-L and advanced carotid atherosclerosis. As a matter of fact, we previously show that increased OPG levels can explain for the association between coronary artery calcification and chronic kidney disease [23]. OPG represents a novel marker of cardiovascular mortality and clinical events in patients with acute myocardial infarction complicated with heart failure [24]. However, there has been little clinical evaluation about the association between circulating OPG and RANK-L and advanced carotid atherosclerosis. As a matter of fact, we previously show that increased OPG levels can explain for the association between coronary artery calcification and chronic kidney disease [23]. OPG represents a novel marker of cardiovascular mortality and clinical events in patients with acute myocardial infarction complicated with heart failure [24].

Furthermore, we previously showed that apoptosis activation in atheromasic plaques may arise from oxidative stress (e.g. hypoxia) or a persistent inflammatory condition [24-29]. It is in fact known that persistent inflammation may worsen atherosclerosis and determine an earlier onset, as a matter of fact young subjects with a mutation on the inflammasome (NLRP3) gene, that result in over-activation of the inflammatory complex, demonstrated signs of atherosclerosis since their early childhood, with increased β stiffness parameter and PWV [30].

The following limitations apply to this study: first, the small sample size; in this respect, these findings need to be confirmed in a larger number of subjects. Furthermore, echo-tracking is a new modality with undoubtful advantages, but with only recent evidence in large clinical studies. In addition, a comparison between conventional measurement of arterial stiffness and local stiffness by QAS was not performed: this could have reinforced our results.
Conclusion

The correlation between RANK-L and local arterial stiffness parameters suggests that arterial wall functional properties may affect plaque evolution toward calcification. However, these findings need to be confirmed in a larger number of subjects. Moreover, the observed activation of inflammatory and apoptotic pathways in heterogeneous plaques together with a clinical history of previous cerebrovascular events in patients having heterogeneous lesions, confirm the predictive role of markers of apoptosis and inflammation regarding to plaque composition and for the identification of the mechanism that could lead to a “culprit plaque”. In light of these results caution should be taken when evaluating those subjects using the humanized monoclonal antibody denosumab, directed to RANK-L, because their atherosclerotic lesions eventually proceed faster through calcification.

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Author Contribution

All authors contributed to: (1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, and, (3) final approval of the version to be published.

References