Journal of Clinical and Experimental Cardiology

Galfo et al., J Clin Exp Cardiolog 2018, 9:2
DOI: 10.4172/2155-9880.1000574

Research Article Open Access

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

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Received date: February 08, 2018; **Accepted date:** February 19, 2018; **Published date:** February 27, 2018

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 endoarterectomy, with homogeneus 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 heterogeneus 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 homogeneus 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

increased stiffness of the arterial wall may be correlated to plaque evolution. In addition, although aortic PWV is an established measure of arterial stiffness, new echo-tracking systems demonstrated to be as reproducible and accurate as conventional method [8-13]. In light of these previous observations, we analyzed the expression profile of the NLRP3 inflammasome, other inflammatory and apoptotic proteins and OPG\RANK-L pathway in carotid plaque specimens, correlating their expression with lipid peroxidation and stiffness parameters by a high definition echo-tracking software. In this study we also aimed at identifying a possible pharmacological target related either to the plaque or to arterial stiffness.

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 endoarterectomy 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 in 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 after 10 min rest. Systolic and diastolic BP values were entered into the system for carotid stiffness parameter calculation. Pulse pressure (PP) was calculated as the difference between systolic and diastolic BP. A simultaneously recorded electrocardiogram was used as a reference to calculate wave transit time.

Optimal images from a long-axis scanning were best achieved by positioning and orienting the probe so that clear and parallel delineation of the intima-media complex at both the anterior and posterior walls could be seen. Six consecutive measurements were performed and only when these consecutive 6 times measurements met the quality standard (quality control shown in green number on the screen during the scanning), the average was taken as the final result for each patient. The measurements and average calculations were automatically done and displayed on the left side of the screen.

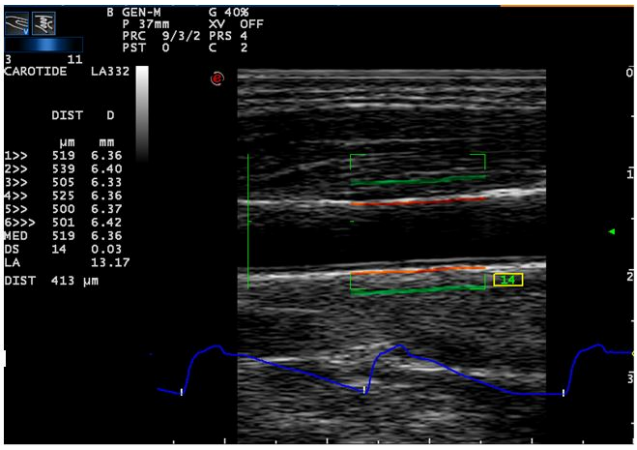

Figure 1: Example of carotid distensibility as obtained by Quality Arterial Stiffness (QAS; MyLab Esaote, Italy) at the level of the Common Carotid Artery (CCA) at about 2 cm proximal to the bifurcation; The movement of carotid walls is tracked in the entire region of interest (green rectangle width 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 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, mm^2/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:

$$PWV=1/\sqrt{\rho} \times DC=\sqrt{D2 \times \Delta p/\rho \times (2 \times D \times \Delta D+\Delta D2)}$$

Where D: Diastolic diameter; ΔD: Change of diameter in systole; DC: Distensibility coefficient; Δp: Local pulse pressure; ρ: Blood density (ρ =1.050 kg/m^3).

Stiffness index β was expressed as:

$$\ln (SP/DP) \times D/\Delta D,$$

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). Protein samples (30 μg) were denatured in reducing buffer (62 mmol Tris/HCl, pH 6.8, 10% glycerol; 2% SDS; 5% mercaptoethanol; 0.003% bromophenol blue) and separated by SDS-PAGE. Membranes were incubated with a primary antibody for NLRP3 (Adipogene, Hamburg, Germany); 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 signal 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-Tetramethoxypropane), 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 Fas (Hs00163653_m1), Fas-L (Hs00181225_m1), Bcl-2 (Hs99999018_m1), caspase-8 (Hs00154256_m1), TNF-α (Hs00174128_m1), TNFRII (Hs00153550_m1), HMGB1 (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 by 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.

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

Table 1: Demographic and clinical characteristics of patients with homogeneous plaque and those with heterogeneous plaque; There was no difference with regard to demographic and clinical (blood pressure values and risk factors) variables between the two groups of patients.

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).

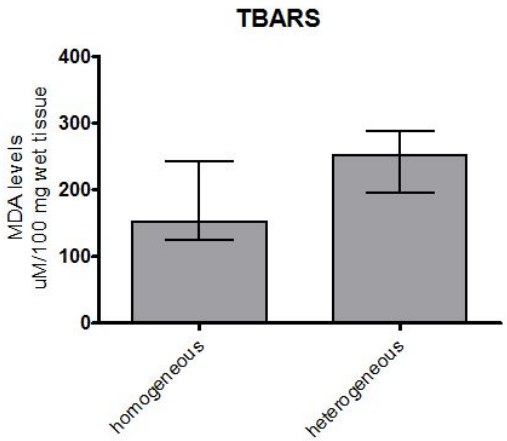

Figure 2: The graph represents lipid peroxidation values in the two groups, Mann-Whitney U-test (one-tailed) showed increased TBARS values in heterogeneous plaques (*p=0.02).

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 homogeneus (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 calcification that 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).

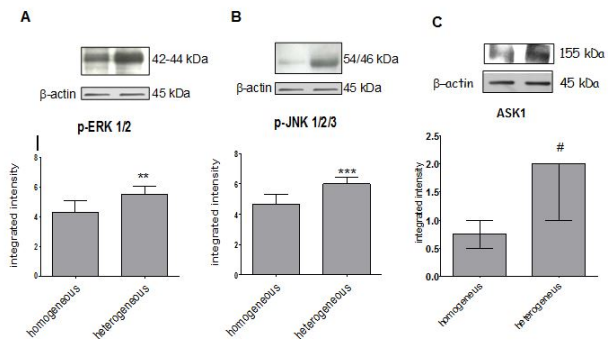

Figure 3: The graph in A represents p-ERK 1/2 western blotting results in the two groups. Mann-Whitney U t-test showed increased levels in heterogeneous plaques (**p=0.03); The graph in B represents p-JNK 1/2/3 western blotting results, showing increased levels in heterogeneous plaques (***p<0.03); The graph in C represents ASK1 western blotting results indicating increased in expression heterogeneous than homogeneous 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.

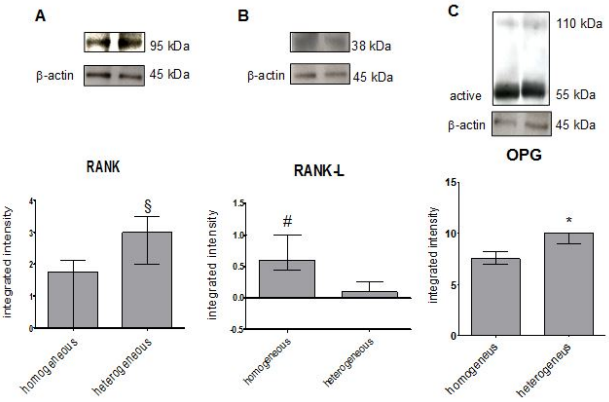

Figure 4: The graphs in A, B, and C represent, respectively, western blotting results of RANK, RANK-L and OPG; Mann-Whitney U t-test showed higher levels of RANK and OPG (§p=0.02 and *p=0.04 respectively) proteins in heterogeneous plaques; RANK-L western blotting results, showing higher levels in homogeneous 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 homogeneous (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.

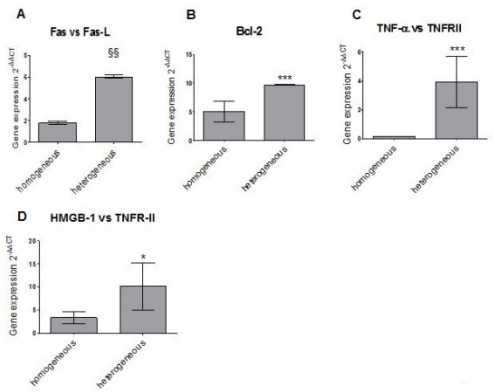

Figure 5: Graphs represent Real Time PCR results; Mann-Whitney U t-test showed higher levels of both A FAS/FAS-L ($^{§§}p=p=0.019$), B Bcl-2 ($^{***}p<0.0001$), C TNFα/TNFR-II ratio ($^{***}p<0.0001$) and D HMGB-1/TNFR-II ratio ($^{*}p=0.04$) in heterogeneous plaques.

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).

Variable	R value	P value
OPG (i.i)	0.511	0.03
RANK (i.i)	0.165	0.29
RANK-L (i.i)	-0.105	0.36
ASK 1 (i.i)	0.393	0.09
NLRP3 (i.i)	0.211	0.24
HMGB (i.i)	0.361	0.11

i.i=integrated intensity

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 ($r=0.511$, $p=0.03$).

Pulse Wave Velocity and protein correlations		
Variable	**R value**	**P value**
OPG (i.i)	0.22	0.23
RANK (i.i)	0.058	0.42
RANK-L (i.i)	0.525	0.03
ASK1 (i.i)	0.301	0.15
NLRP3 (i.i)	-0.073	0.4
HMGB (i.i)	0.166	0.29
β-index and protein correlations		
Variable	**R value**	**P value**
OPG (i.i)	0.03	0.46
RANK (i.i)	0.078	0.39
RANK-L (i.i)	0.514	0.03
ASK1 (i.i)	0.167	0.29
NLRP3 (i.i)	-0.248	0.2
HMGB (i.i)	0.032	0.45
i.i= integrated intensity		

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).

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 stiffness 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 Ca^{2+} by Vascular Smooth Muscle Cells (VSMC), and also by a de-regulation of the RANK/RANK-L/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 levels of OPG were detected in atheromas, and particularly in the heterogeneous plaques with initial signs of calcification [25], in agreement with our previous data we observed increased OPG levels in heterogeneous plaques. Osteoprotegerin acts as a decoy receptor for RANK-L, that resulted reduced in heterogeneous plaques, and this probably led to an indirect increase in the natural receptor RANK in the same type of plaques. The deregulation of this axis could also result from chronic inflammatory mechanisms, thus we evaluated the expression of the NLRP3 inflammasome and MAPKs, in fact activation of the inflammatory pathway may represent an important milestone in plaque progression, that further increase oxidative and mechanical stress in the arterial wall. The oxidative stress, evaluated by TBARS was significantly increased in heterogeneous atheromas, in the same specimens we found increased levels of the active forms of ERK and JNK, indicating an active inflammatory process in these lesions. It is known that ERK activation in atherogenesis regulates many inflammatory and vascular cells functions, promoting, for example, leukocyte migration [26] and may be also involved in plaque vulnerability [19]. With regards to JNK isoforms, both *in vivo* and *in vitro* studies showed that active JNK-2 is able to mediate foam cells formation in atherogenesis [27], and to reduce endothelial dysfunction induced by hypercholesterolemia or by oxidative stress [28]. In our heterogeneous specimens we observed increased JNK 1/2/3 levels by western blot analysis, however, the specificity of the antibody used was not sufficient to individually identify JNK-2.

The analysis of NLRP3 in atherosclerotic plaques revealed a strong presence of this marker in both plaque types, suggesting an activation of "sterile inflammation" in atheromas, without significant differences among groups.

Another pathway that has been demonstrated to be involved in the development of atherosclerotic plaques is the apoptosis, in fact, the oxidant microenvironment reduce macrophages clearance of cell debris, which accumulate in the lipid core increasing proinflammatory cytokines production [29]. As the MAPKs begin the inflammatory cascade within the cells, another kinase that cross-talks with MAPKs is able to trigger the apoptosis signalling and this kinase, known as apoptosis signal kinase (ASK1), is also able to stimulate early and late apoptotic cytokines as TNF-α and HMGB-1.

In our specimens we investigated by western blot the endogenous levels of ASK1 and we found an higher level in heterogeneous than homogenous, on the other side HMGB-1 levels were not particularly elevated neither showed differences between the two groups. However, gene expression analysis for HMGB-1 and TNF-α showed a significant increase of both mRNAs, especially when compared to the main TNF receptor, TNFRII, suggesting that the apoptotic trigger was strong and persistent in the heterogeneous plaques. When considering the extrinsic apoptotic pathway by the relative gene expression levels of the two main activators Fas and Fas-L we observed also in this case that heterogeneous plaques showed an increased expression of these specific mediators. As a further confirmation of an active apoptotic machinery, the relative gene expression of Bcl-2 demonstrated an higher expression of this anti-apoptotic molecule, most likely due to a compensatory mechanism in the heterogeneous plaques, to limit the intra-plaque degeneration. All these findings could be related to the higher levels of lipids peroxidation and oxidative stress identified in the heterogeneous plaques.

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.

Acknowledgements

The authors are thankful to Dr. Stefano Pedri, B.Sc., Global Medical Affairs, Esaote S.p.A., Florence, Italy, for its technical assistance.

Funding

This research was funded by departmental funding only.

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.

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