Virtual Histology-Intravascular Ultrasound as a diagnostic alternative for morphological characterization of carotid plaque: comparison with histology and High-Resolution Magnetic Resonance findings

Poster No.: C-0841  
Congress: ECR 2012  
Type: Scientific Exhibit  
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Keywords: Interventional vascular, Head and neck, Arteries / Aorta, CT-High Resolution, MR-Angiography, Percutaneous, Arterial access, Stents, Angioplasty, Arteriosclerosis, Embolism / Thrombosis, Tissue characterisation  
DOI: 10.1594/ecr2012/C-0841

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Purpose

The primary target was to validate VH-IVUS as a diagnostic tool for plaque characterization through in vivo characterization of carotid artery plaques by correlation to ex vivo histological specimen. The secondary target was to compare in vivo VH-IVUS with HR-MR imaging.
Methods and Materials

Ex vivo specimen preparation

Data was acquired from six human carotid arteries explanted previous post mortem familial consent and after approval from our hospital's ethic committee. Six single carotid arteries were explanted from six consecutive male patients with a mean age of 72 ± 9.64 years, with known history of cerebral ischemia, either Transient Ischemic Attack (TIA) or stroke.

Patients with a previous endovascular or surgical revascularization were excluded. The time interval between death and specimen explantation ranged from 8 to 12 hours. VH-IVUS imaging was performed within 48 hours from death.

Specimens were preliminarily fixated in a 10% formalin solution and were conserved at room temperature before the study.

Three sutures placed on outer surface of each artery were used as a reference to guarantee the correct overlap, with an exact "tomographic" correlation, between ultrasonographic images and histological specimens. The three sutures, differently coloured, were placed on the proximal third of the common carotid artery and on the distal thirds of external and internal carotid arteries.

The arteries were explanted from the common carotid artery ostium to the distal extracranial segment, including approximately 40 mm of the surrounding adipose and muscular tissues in order to maintain a supporting structure for the vessels.

After mounting the specimens on the dissecting tray, they were orientated and were positioned in a container filled with a 10% formalin solution or saline solution, as described above. A compressed air system, designed at the Biophysics Department of Tor Vergata University, was used to allow phosphate buffered saline (PBS) flow through the vessels.

The arterial branches were clamped to allow an intraluminal pressure of 100 mmHg, as described in other studies [9-10].

IVUS and histological data acquisition

Ultrasonographic data was acquired using a 20 MHz IVUS apparatus (Eagle Eye, Volcano Therapeutics, Rancho Cordova, CA). After introducing the IVUS catheter into each artery, sectional images of the plaques were obtained using a 1.0 mm/sec pull-back system. For this study, an area of stenosis > 60% for each section evaluated by IVUS was considered as significant.

VH-IVUS data was successively stored on a personal computer for further off-line analyses. Each specimen was then immediately immersed in saline solution and conserved in sealed 15-20 ml vials for the 2 hours following the IVUS session. The specimens were maintained at room temperature to avoid a phase transition of the constituents. A quick-freeze of the vials was not performed in order to guarantee the integrity of the specimen and to avoid a distortion of the tissues caused by the presence of internal calcifications.

The carotid artery specimens were fixated in buffered 10% formalin solution for 24 hours. The methods for specimen preparation have been previously described [11-12].
A CT examination of the plaque was performed for the evaluation of calcium presence, in order to perform a correlation with data from HR-MR and VH-IVUS. A subsequent decalcification of the specimens was performed in order to cut the plaque into segments. The specimens were, then, sectioned perpendicularly to their longitudinal axis with a 3 mm thickness, were immersed in paraffin and stained by hematoxylin-eosin or Movat pentachrome stain. Each section was numbered sequentially from the proximal to the distal segment of the internal carotid artery in order to reconstruct the plaque in its whole length.

**Histological Analysis**

The histological sections were analyzed by a histopathologist, unaware of the results obtained by VH-IVUS. The analysis was performed according to the classification of atherosclerotic lesions of the American Heart Association (AHA) Council on Atherosclerosis [13]. Four types of plaque components were identified: collagen, fibrolipid core, calcium and necrotic tissue. Areas with predominant collagen content were assimilated to fibrous tissue, while areas with lipid content predominating over collagen to fibro-lipid tissue. Regions containing residues of cholesterol, foam cells and micro-calcifications were defined as necrosis. After identifying the different areas within the plaque, digital images were created (72 dpi, dot per inch) using an artwork software (Adobe Photoshop version CS3, Adobe Systems Inc., CA, USA) setting a specific color to each of the four tissues considered. The number of pixels (picture elements) of each of the four colors was calculated and, accordingly, the percentage of the different tissue components was defined in all plaques. The percentages obtained were considered as the reference standard for the study.

**VH-IVUS - Histology correlation**

The exact correspondence between the histological sections and the VH-IVUS images was determined using the sutures which were placed on the outer surface of each artery, using the morphology of each section to obtain an acceptable section match. The VH-IVUS and the digitalized histopathological images (png format) were optimized to raster images and normalized to a standard dpi format (72 dpi). The VH-IVUS slices used for the analysis (8-10 per specimen) were selected accordingly to the existence of regions involved by critical plaque that were identified on the digitalized images by an expert histopathologist.

Using a pixel-by-pixel segmentation, the VH-IVUS Lab software (Volcano Therapeutics Inc.) elaborated 4 tissue maps for each selected image: fibrous tissue, fibro-lipid tissue, necrosis and calcium.

The selected digital histopathological images were then processed through their manual segmentation into four different tissue segments by an expert histopathologist unaware of the VH-IVUS findings, using an artwork software (Adobe Photoshop, version CS3 Adobe Systems Inc., CA, USA) [Fig. 1]. Finally, the percentage of each tissue component was defined according to the number of pixels contained in the different segments [14].

**In-vivo patient population**
Twelve consecutive patients (8 males, 4 females, mean age of 75 ± 6.33 years), candidates for Carotid Artery Stenting were included in this study. The enrolled patients were considered symptomatic after neurological evaluation. All of them were underwent to MRI brain examination with intracerebral angiographic sequences within three month from the last clinical symptom.

The degree of the stenosis ranged between 60 and 80 % and were identified by eco-color-Doppler ultrasound and Computed Tomography Angiography. All patients had undergone a neurological evaluation and a brain CT or MR scan before endovascular treatment.

In-vivo HR-MRI

The stenoses were localized by Duplex Doppler Ultrasound ultrasonography and their exact position was localized on the skin to allow a precise positioning of the MR microcoil. Patients were evaluated within 15 hours before the endovascular procedure.

Exclusion criteria consisted in the presence of general contraindications to MR or to gadolinium contrast medium administration.

The HR-MRI study protocol was performed using a 1.5 Tesla apparatus (Gyroscan Intera, Philips, Best, The Netherlands), with a maximum gradient strength and slew rate of respectively 33mT/m and 80 mT/m/ms), and a microscopy radiofrequency surface coil (Microscopy 47mm, Philips, Best, The Netherlands) positioned at the carotid bifurcation.

Patients were examined in supine position using a head support in order to reduce movement artifacts. The head was slightly turned on the opposite side of the examined carotid artery to expose the carotid bifurcation, and to prevent sterical constraints from the mandibular bone and sternocleidomastoid muscle.

Acquisitions were triggered with the heart frequency, which was monitored during the whole examination using an electrocardiograph (cardiographic gating VCG), in order to reduce artifacts related to arterial sphygmic movements.

The carotid bifurcation was identified using T1 FFE 3D sequences with the Time of Flight (TOF) technique on the axial plane, with the quadrature body coil (Q-body coil) used for signal reception. The automatic rotational MIP post-processing elaboration, on the coronal and sagittal planes, allowed the precise identification of the site of the atheromatous lesion with a total acquisition time of 2' 30".

The 2D multiparametric morphological images were placed on the MIP images on the axial plane perpendicularly to the vessel's main axis: T1w BB TSE (TE/TR 18/2 beats per interval R-R; 90° flip-angle; 90/100% FOV/RFOV; 208/512 matrix/reconstruction; 1.5/0.5 slice thickness/gap; 2 NEX; 4' 6" scan time); T2w TSE BB (TE/TR 60/2 beats per interval R-R; 90° flip-angle; 90/100% FOV/RFOV; 208/512 matrix/reconstruction; 1.5/0.5 slice thickness/gap; 2 NEX; 2'48" scan time); PDw TSE (TE/TR 15/2 beats per interval R-R; 90° flip-angle; 90/100% FOV/RFOV; 208/512 matrix/reconstruction; 1.5/0.5 slice thickness/gap; 2 NEX; 2'24" scan time).

2D and 3D T1 FFE TOF bright-blood images were then acquired on the axial plane obtaining "luminographic" images that were than fused with the data obtained from the vessel wall analysis.

Finally, T1w TSE BB images were obtained 7-10 minutes after intravenous infusion of gadolinium-DTPA.
Total examination time, including patient positioning, was approximately 40-45 minutes.

VH-IVUS Imaging protocol and Carotid Artery Stenting (CAS)

An informed patient consent was obtained for each patient prior to the procedure. A 5-day anti-aggregation therapy with acetilsalicilic acid (100 mg / day) and clopidogrel (75 mg / day) (Plavix® - Bristol-Myers Squibb/Sanofi Pharmaceuticals Partnership Bridgewater) was administered before the procedure.

In each procedure, a bolus of 5000 IU of heparin was administered intravenously to maintain the Active Coagulation Time (ACT) between 200 and 250 seconds after obtaining transfemoral retrograde access. During each procedure, a cerebral protection device (Epi-filter, Boston Scientific) was placed before VH-IVUS examination and stent deployment. The Gray-Scale and VH-IVUS evaluations were performed using a 20-MHz IVUS (Eagle Eye, Volcano Therapeutics, Rancho Cordova, CA) probe. The IVUS catheter was washed with saline solution prior to its use. The internal carotid artery was selectively catheterized using the IVUS catheter. After optimization of the gain, the IVUS catheter was retrieved from the distal segment of the internal carotid artery at a speed of 1.0 mm/sec using a motorized pull-back device. The IVUS scan during the pull-back maneuver included at least one proximal (vessel ostium) and one distal marker to allow a comparison between the VH-IVUS and MR images. Examinations were recorded on a DVD.

Each patient underwent a brain MR scan 2 hours after CAS.

HR-MR and VH-IVUS data correlation

Two expert radiologists (M.C. and S.F.) determined the adequacy of the selected images for the correlation, applying the criteria of the American College of Cardiology / American Heart Association (ACC/AHA) consensus statement on IVUS (External Elastic Membrane - EEM visibile for at least 270°) [15]. Both radiologists evaluated the presence of fibro-lipid tissue, fibrous tissue, necrosis or calcium in the plaque, first independently and then jointly in order to rule out eventual interpretational doubts.

The specific tissue components were defined as follows: fibro-lipid tissue as a relatively hypoechocic area compared to the adventitia, fibrous tissue as a hyperechoic area without acoustic shadow and calcium as a hyperechoic area with acoustic shadow. Afterwards, the manual elaboration of the margins of each axial image was performed to obtain VH images.

The intra-luminal margin and the EEM were then identified. The two radiologist then selected the images for the VH-IVUS/HR-MR correlation. The correspondence between the images of the two methods was determined according to the distance from the vessel's ostium and the presence of identical morphological characteristics. Evaluating the morphology of the plaque and using the jugular vein as an orientation landmark, correct orientation and overlapping between the VH-IVUS and HR-MR images was achieved.

All HR-MR images were converted to a digital format (tiff) using a dedicated console, preserving the original signal intensity of the pixels. The exact match between VH-
IVUS and HR-MR images was obtained using as a reference the distance from the vessel's ostium and the intrinsic morphological characteristics of the vessel sections. The exact position of the IVUS probe, visible also on the DSA images due to its radiopacity, was determined according to the known speed (1.0 mm/sec) of the motorized pull-back system. HR-MR and VH-IVUS images with higher atheromatous involvement were selected for the correlation analysis.

Selected HR-MR and VH-IVUS images, optimized to raster graphics images and converted to a standard tiff format (72 dpi), were examined by both radiologists with the same criteria used for VH-IVUS-imaging processing, using a pixel by pixel segmentation. Images were merged into four tissue classes: fibrous tissue, fibro-lipid tissue, necrosis and calcium using the same method used for the histopathological images, as previously described. These classes were correlated to the plaque components identified by VH-IVUS: fibrous tissue (green), fibro-lipid tissue (yellow), necrosis (red) and calcium (white). To establish the presence of a specific plaque component, multiple adjacent pixels had to show the same signal intensity.

Once digital images were obtained and the number of pixels for each plaque component was calculated, the exact percentages of the 4 plaque components were determined [Fig.2].

**Statistical analysis**

The percentages of the various plaque components provided by the observers were compared for each method. Concordance between methods (histology vs VH-IVUS and VH-IVUS vs HR-MR) and inter-observer variability were calculated using Cohen's # test for concordance [16]. The value included in a range between 0.61 and 0.80 was considered the index of a significative concordance [17].

Comparison of histology vs VH-IVUS and VH-IVUS vs HR-MR reported a value of concordance of 0.80 and 0.76, showing a significative concordance in each method.

Sensitivity and specificity of VH-IVUS were determined considering histology as the reference standard. Statistical significance was established using the Fisher Test at P value < 0.05.

Statistical analysis was performed using the SPSS version 14.0 software (SPSS 19, Chicago, Ill). All statistical analyses were performed by two authors and a statistician.
Fig. 1: Histological image (a). Digitalized image processed through its manual segmentation into 4 different tissue segments by an expert histopathologist (b-d): brown: EEM; yellow: fibro-lipid tissue; green: fibrous tissue; white: calcium; red: necrosis. VH-IVUS showed a good correlation with histology especially with regards to the differentiation of the fibrous and fibro-lipid tissues (c).

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**Fig. 2:** Fibro-lipid plaque with interruption of the fibrous cap reported on the PDw (a) and T2 (b) image, with a small ulceration, that can be easily seen on the corresponding VH image (e). The T1w GD sequences show enhancement of fibrous cap , mainly in the site of the rupture (c). The normalized MR image (d) shows significant correlation with the VH findings (e)

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Fig. 3: Carotid bifurcation specimen example.

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**Fig. 4:** Ultrasonographic data acquired using a 20 MHz IVUS apparatus. Sectional images of the plaques were obtained using a 1.0 mm/sec pull-back system.

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**Fig. 5:** The four tissue maps of VH-IVUS: fibrous tissue, fibro-lipid tissue, necrosis and calcium.

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Fig. 8: HR-MRI: DPw (a) and T2w sequence (b) show a fibrolipidic plaque; T1wGD sequence (c) show inflamed fibrous cap enhancement and marginal necrotic plaque component, well documented in the corresponding VH-IVUS image (e) with a good correlation with the normalized MRI image (d).

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**Fig. 7:** Fibrolipidic plaque with interrupted fibrous cap, evident either in DPw (a) and in T2w sequences (b) The ulceration is evident also in VH-IVUS image (d). T1w GD show a focal enhancement in correspondence of the fibrous cap superficial ulceration (c). The normalized MR image (e) presents a significant concordance with the VH-IVUS image (d)

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**Fig. 6:** In-vivo IVUS evaluation of carotid plaque imaging before and after stent release

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Fig. 9: HR-MRI: DPw (a) and T2w (b) sequences show a fibrous plaque with minimum lipidic content; T1wGD (c) sequences show no pathologic enhancement. Corresponding VH-IVUS image (e) shows a good correlation with the normalized MR image (d), with a slight overestimation of the necrotic area.
Results

VH IVUS - histology correlation

In the group of patients who underwent to CEA treatment, forty-two images of the 54 available sections were obtained and used for correlation (8-10 sectional images obtained by 6 carotid arteries) between VH-IVUS and histology. Twelve images were excluded from the study due to plaque fragmentation during either surgical endarterectomy of histological processing.
Quantitative analysis of different plaque components revealed a good concordance (0.80) between the two methods (95% CI 0.69 to 0.92).
Sensitivity of VH-IVUS in characterizing fibrous tissue, fibro-lipid tissue, calcium and necrosis, considering histology as the reference standard, resulted respectively 100.0%, 94.2%, 84.1%, and 67.1%, while its specificity was respectively 99.2%, 84.3%, 97.4%, and 99.2%.
Diagnostic accuracy of VH-IVUS for concordance with true histology of different plaque components resulted 99.4% for fibrous tissue, 85.9% for fibro-lipid tissue, 71.4% for calcium and 83.4% for necrosis [Table 1].

HR-MR - VH-IVUS correlation

During in-vivo IVUS evaluation no clinical complications were observed. No major or minor cerebrovascular ischemic events occurred in clinical and instrumental examination after CAS procedure.
In all cases it was possible to obtain a complete characterization of plaque morphology.
Correlation between the two radiologists in VH-IVUS imaging processing and in HR-MR imaging segmentation resulted respectively good (0.76) and excellent (0.92).
Comparison between HR-MR and VH-IVUS was performed on 27 images. Concordance between the two methods resulted 0.76 (95% CI 0.69 to 0.92). Sensitivity of VH-IVUS in the characterization of fibrous tissue, fibro-lipid tissue, calcium and necrosis, considering HR-MR as the reference standard, resulted respectively 86.1%, 93.7%, 89.3% and 65.4%; specificity resulted respectively 84.3%, 97.5%, 99.2%, and 98.1%. Diagnostic accuracy resulted respectively 85.3%, 95.2%, 90.2% and 82.0%, [Table 2]. In 6 images, HR-MR identified areas of contrast-enhancement in the fibrous cap.
Conclusion

We revealed a strong correlation between data obtained from VH-IVUS evaluation with HR-MR and histological examination. This study, thus, supports the validity of VH-IVUS in assessing carotid artery plaque raising the possibility to evaluate carotid plaque morphology during therapeutic procedures. Assessment of necrotic plaque components, much represented in unstable, inflamed, at high embolic risk plaques, resulted not optimal using VH-IVUS. These results, however, needs further development in order to data similar to histological findings.
### Table 2: VH IVUS - Histology correlation † Concordance : 0.80 (95% CI 0.69 to 0.92)

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Table 1: HR-MR - VH-IVUS correlation † Concordance : 0.76 (95% CI 0.69 to 0.92)

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<th>Diagnostic Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
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<tr>
<td>Fibrous tissue</td>
<td>85.3 %</td>
<td>86.1 %</td>
<td>84.3 %</td>
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<tr>
<td>Fibro-lipidic tissue</td>
<td>95.2 %</td>
<td>93.7 %</td>
<td>97.5 %</td>
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<td>Calcium</td>
<td>90.2 %</td>
<td>89.3 %</td>
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<td>Necrosis</td>
<td>82.0 %</td>
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References


