Unreliable Assessment of Necrotic Core by Virtual Histology

Intravascular Ultrasound in Porcine Coronary Artery Disease

Troels Thim, MD; Mette Kallestrup Hagensen, MSc; David Wallace-Bradley, MSc; Juan F. Granada, MD; Greg L. Kaluza, MD, PhD; Ludovic Drouet, MD, PhD; William P. Paaske, MD, DMS; Hans Erik Bøtker, MD, PhD, DMS; Erling Falk, MD, DMS

Background—Intravascular ultrasound–derived virtual histology (VH IVUS) is used increasingly in clinical research to assess composition and vulnerability of coronary atherosclerotic lesions. However, the ability of VH IVUS to quantify individual plaque components, in particular the size of the destabilizing necrotic core, has never been validated. We tested for correlation between VH IVUS necrotic core size and necrotic core size by histology in porcine coronary arteries with human-like coronary disease.

Methods and Results—In adult atherosclerosis-prone minipigs, 18 advanced coronary lesions were assessed by VH IVUS in vivo followed by postmortem microscopic examination (histology). We found no correlation between the size of the necrotic core determined by VH IVUS and histology. VH IVUS displayed necrotic cores in lesions lacking cores by histology.

Conclusions—We found no correlation between necrotic core size determined by VH IVUS and real histology, questioning the ability of VH IVUS to detect rupture-prone plaques, so-called thin-cap fibroatheromas. (Circ Cardiovasc Imaging. 2010;3:384-391.)

Key Words: intravascular ultrasound ■ virtual histology ■ vulnerable plaque ■ coronary disease ■ animal model

Plaque rupture is responsible for ≈75% of all myocardial infarctions, fatal as well as nonfatal.¹² Therefore, the most important vulnerable plaque type is the rupture-prone plaque, which is also known as a thin-cap fibroatheroma (TCFA). Plaque rupture requires the presence of a lipid-rich necrotic core, and the risk of plaque rupture increases with necrotic core enlargement.³⁴

Editorial see p 348

Clinical Perspective on p 391

Reliable TCFA identification and plaque rupture prediction may enable plaque rupture prevention by local intervention.⁵ However, this concept is unproven, and we lack documentation for reliable prospective identification of rupture-prone plaques.

Intravascular ultrasound (IVUS) interrogates the artery wall, and an IVUS-derived virtual histology (VH IVUS) definition of TCFA relying on necrotic core size has been proposed.⁶ By this definition, plaques with percent atheroma volume ≥40% and necrotic core ≥10% without evident overlying fibrous tissue are classified as TCFA lesion. However, VH IVUS validation studies on human coronary arteries ex vivo have focused solely on the detection of particular tissue types in limited homogeneous regions of interest.⁷⁻⁹ This validation method was also used in a recent in vivo study.¹⁰ VH IVUS has never been validated for determination of absolute or relative area of any particular tissue component in human coronary arteries. Experimental studies in porcine coronary arteries¹¹ and rabbit aortas¹² focusing on the capability of VH IVUS to determine the relative size of the 4 tissue components included in the VH IVUS algorithm have produced conflicting results.

We tested for correlation between VH IVUS necrotic core size and necrotic core size by histology in adult minipigs, in which human-like coronary atherosclerosis develops spontaneously and included both native and balloon-accelerated coronary lesions in the study.

Methods

The experimental procedures were approved by the Danish Animal Experiments Inspectorate.
Porcine Coronary Artery Disease Model
Ten castrated male minipigs with low-density lipoprotein receptor mutation, downsized from the original Rapaz farm pig,13–15 were used in the present study. To accelerate coronary artery disease development, the minipigs (mean age, 9 months) were fed an atherogenic diet for 18 weeks, containing 2% cholesterol, 20% lard, and 1.5% cholate (wt/wt; TestDiet 5837V). After 4 weeks on this diet, 2 coronary artery segments per pig were mechanically injured with an oversized angioplasty balloon to accelerate local plaque development. After 18 weeks on the atherogenic diet, we performed in vivo coronary VH IVUS acquisition and then performed postmortem coronary microscopic examination.

VH IVUS Acquisition and Analysis
VH IVUS images were recorded on a Volcano imaging system with a 20-MHz Eagle Eye Gold IVUS imaging catheter (Volcano Therapeutics Inc, Rancho Cordova, Calif). Before acquisition, heparin was administered intravenously. Intracoronary nitroglycerin was administered before every pullback. After positioning the IVUS catheter distal to the lesion of interest, an automated pullback was performed at 0.5 mm/s. Two coronary arteries per minipig were interrogated. The location of the IVUS catheter was monitored by fluoroscopy and recording of side branches seen during IVUS imaging. Continuous heart rhythm monitoring was used to gate IVUS acquisition.

VH IVUS is spectral analysis of radiofrequency ultrasound signals. The analysis was performed offline (pVH ver2.2; Volcano Therapeutics Inc). Automatically detected lumen and vessel border contours were manually corrected as recommended and tissue composition color-codes were generated by the software with 4 color codes: red for necrotic core, light green for fibrofatty tissue, dark green for fibrous tissue, and white for dense calcium. The software also reported absolute areas for plaque and the 4 tissue components and the relative contribution of the 4 tissue components to plaque area.

Histology
Immediately after VH IVUS interrogation, the heart was excised and the coronary arteries were perfusion-fixed with 4% formaldehyde at 100 mm Hg for 1 hour and then immersion-fixed. Noting the anatomic landmarks from angiography and IVUS, the coronary arteries were sectioned at 4-mm intervals, paraffin-embedded, sectioned, and stained for microscopic examination (histology). The luminal area and the area within the internal elastic lamina were measured by planimetry on digital photomicrographs (ImageJ, NIH) and plaque area was calculated (the difference between the 2 areas). For sections containing a necrotic core, the absolute necrotic core area was measured by planimetry and the relative necrotic core size was calculated (necrotic core area/plaque area). We defined necrotic cores as areas in which the extracellular matrix was lacking (total loss of collagen by picrosirius red staining) and replaced by dead cells and cellular debris (no or fragmented nuclei by hematoxylin and eosin staining). Compared with human necrotic cores, cholesterol crystals were relatively rare, as expected.16

VH and Histology Alignment and Comparison
VH IVUS frames and histology slides were carefully aligned, based on a thorough examination of angiographies and IVUS pullbacks and macroscopic and microscopic evaluation. The alignment was aided by fiduciary points such as side branches and the clearly demarcated balloon injury sites and by the fact that spontaneous coronary artery disease was not diffuse but multifocal. We only investigated lesions with an intimal area larger than 1 mm² by histology because ruptured lesions are relatively large, and predominantly larger lesions are interrogated with this technology clinically.17 In segments containing a lesion with an intimal area >1 mm² by histology, the section with the largest intimal area was used in the analysis and compared with the VH IVUS frame with the largest plaque area within that segment.

Because necrotic core size is considered a key determinant of plaque vulnerability and is included in the VH IVUS TCFA definition, we tested whether VH IVUS necrotic core size and necrotic core size by histology were correlated.

To investigate the possible influence of correct tracing of the vessel border on VH IVUS tissue mapping of the innermost and critical part of the lesion, we reanalyzed 1 plaque with the vessel border deliberately moved inward into the plaque and outward into the adventitia. Similarly, to investigate the effect of correct tracing of the luminal border, we deliberately moved it into the plaque.

Statistical Analysis
Summarized data are given as mean (standard deviation, SD). The necrotic core size data had a non-normal distribution even after different transformations, including the log transformation. Therefore, correlations were tested with nonparametric correlation analysis with calculation of Spearman ρ and the corresponding probability value (Stata/IC 10.1, StataCorp LP, College Station, Tex). A value of P<0.05 was considered statistically significant. Observations within the same minipig may not be strictly independent observations. By examination of plots, the data set has been examined for dependency of observations within pigs. Observations within pigs were not dependent and therefore the observations were treated as independent observations in the analysis.

Results
Minipig Model
The minipigs were 13 months (SD, 2 months) old and weighed 42 kg (SD, 17 kg) at the end of the study. The plasma total cholesterol levels were 6.0 mmol/L (SD, 0.3 mmol/L) on standard diet at baseline and peaked at 21.6 mmol/L (SD, 1.3 mmol/L) on the atherogenic diet. The minipigs developed spontaneous coronary lesions outside balloon-injured sites only in the proximal coronary segments. The balloon injuries were performed in more distal segments of the coronary arteries increasing the number of lesions available for investigation. One minipig died during follow-up, so 9 pigs were available for VH IVUS interrogation.

Histology
Among lesions interrogated with VH IVUS, the criterion of intimal area >1 mm² by histology was met by 18 lesions. These lesions had a mean intimal area of 3.3 mm² (SD, 1.5 mm²). Six lesions had developed spontaneously and 12 were balloon accelerated. The 6 spontaneous lesions all contained necrotic cores. Of the 12 balloon-accelerated lesions, 6 contained necrotic cores. In the 12 lesions with necrotic cores, the mean necrotic core size was 0.8 mm² (SD, 0.9 mm²), corresponding to 21% (SD, 23%) of plaque area.

VH IVUS
For these 18 selected lesions, the corresponding VH IVUS frames were identified. The VH IVUS results are summarized in the Table.

Correlation Between VH IVUS and Histology
There was no correlation between necrotic core size determined by VH IVUS and histology, neither in absolute nor relative measures (Figure 1). VH IVUS displayed necrotic cores in 5 of 6 of the lesions without necrotic cores.

Illustrative examples of VH IVUS and histology comparisons are given in Figures 2 and 3, with both IVUS frames and histology slides viewed from the proximal side. Neither
the size nor the intraplaque location of the necrotic core matched.

In Figure 2, the plaque contained necrotic core areas that were largely classified as fibrofatty by VH IVUS, and the areas classified as necrotic areas by VH IVUS corresponded largely to fibrotic areas. In Figure 3, the lesion consisted of rather homogeneous, collagen-rich fibrous tissue without necrotic core or calcification. Nevertheless, VH IVUS classified a large portion of this lesion as necrotic core and dense calcium.

Discussion
We found no correlation between necrotic core size determined by VH IVUS and histology. This finding substantiates our previous results obtained in another porcine model of complex coronary lesions. Considering the widespread clinical and research applications of VH IVUS, our results may appear surprising. However, in 1 study comparing acute coronary syndrome and stable angina patients, positive remodeling and acute presentation were associated with smaller VH IVUS necrotic core areas, which is highly contradictory to recent coronary computed tomography data and the common conception of the vulnerable plaque based on numerous pathological studies.

Alignment of VH IVUS Frames and Histology Slides
When comparing images obtained by 2 different imaging technologies such as VH IVUS and microscopy, considering whether the VH IVUS frames and histology slides were properly matched is essential. We used fiduciary points from angiographies, IVUS, and macroscopic and microscopic examination to carefully juxtaposition VH IVUS with histology. This methodology was also used in previous in vivo validation studies. The use of fiduciary points on angiographies and IVUS has also been used when comparing 2 different IVUS pullbacks in clinical studies.

Necrotic Core Definition
During necrotic core formation, the extracellular matrix is degraded, and the “unstable” core is characterized by the total lack of supporting collagen. We used a definition and detection method similar to those used in human studies by real histology, necrotic cores were defined as areas in which the extracellular matrix was lacking (total loss of collagen by picrosirius red staining) and replaced by dead cells (fragmented or no nuclei by hematoxylin and eosin staining) and lipid-rich cellular debris. As in humans, microcalcifications were not always present and not required to define a necrotic core. This appears to differ from the definition used in VH IVUS.

Impact of Vessel Border Tracing on VH IVUS Tissue Mapping
The vessel border on IVUS is the border between the tunica media and adventitia and hence corresponds to the external elastic lamina. In the presence of a normal media, this border forms a well-defined leading edge on IVUS. When the media is disrupted by balloon injury or degraded and atrophic beneath atherosclerotic lesions, the vessel border is not always well defined on IVUS. We tested how sensitive VH IVUS was to the location of the vessel border (Figure 4). Moving the vessel border inward or outward did not change the VH IVUS composition analysis of the innermost and critical part of the lesion. VH IVUS displayed necrotic cores (red) and calcifications (white) in a fibrous and rather homogeneous neointimal lesion devoid of necrotic cores and calcifications by real histology (Figure 3).

Table. VH IVUS Results From 18 Porcine Coronary Lesions

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumen area, mm²</td>
<td>7.3</td>
<td>1.4</td>
<td>5.4</td>
<td>10.7</td>
</tr>
<tr>
<td>Vessel area, mm²</td>
<td>15.0</td>
<td>3.6</td>
<td>10.0</td>
<td>22.5</td>
</tr>
<tr>
<td>Plaque area, mm²</td>
<td>7.7</td>
<td>2.9</td>
<td>4.3</td>
<td>14.6</td>
</tr>
<tr>
<td>Plaque burden, %</td>
<td>50.4</td>
<td>8.1</td>
<td>35.0</td>
<td>65.0</td>
</tr>
<tr>
<td>Necrotic core (red) area, mm²</td>
<td>0.7</td>
<td>0.8</td>
<td>0.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Necrotic core (red) relative area, % of plaque area</td>
<td>13.8</td>
<td>13.5</td>
<td>0.0</td>
<td>57.0</td>
</tr>
</tbody>
</table>

Figure 1. Plot of necrotic core size determined by VH IVUS and histology. For both absolute (A) and relative (B) necrotic core areas, no correlation was found.
Impact of Luminal Border Tracing on VH IVUS Tissue Mapping

We also tested how sensitive VH IVUS was to correct tracing of the luminal border (Figure 5). Moving the luminal border into the plaque changed the VH IVUS tissue mapping of the innermost and critical part of the lesion. Areas that VH IVUS displayed as necrotic cores (red) in the plaque middle with the correct luminal border were largely displayed as fibrous at the incorrect luminal boundary. Composition analysis of the deeper parts of the plaque did not change. Moving the luminal border thereby influenced tissue mapping at the luminal boundary but did not lead to progressing tissue mapping changes into the deepest parts of the plaque.

IVUS Tissue Characterization: Gray Scale Versus VH

For gray scale IVUS, it is generally agreed that echolucent plaque areas represent collagen-poor areas with high lipid content within a preserved (lipid pool) or degraded (necrotic core) extracellular matrix. Increasing fibrous content gradually increases echogenicity to a point where very dense fibrous tissue can be hard to discern from calcification. Therefore, gray-scale IVUS offers tissue characterization to some extent.

The purpose of VH IVUS is to improve tissue characterization capability, that is, to identify a necrotic core with higher certainty than offered by gray-scale IVUS. However,
our data suggest that VH IVUS may miss the necrotic core when calcifications in the necrotic core are not present or not detected. Additionally, our data suggest that VH IVUS may interpret fibrosis as necrotic core, which is probably less likely with gray-scale IVUS. These issues may be related to assumptions about necrotic core calcifications reflected in the original necrotic core terms “calcified necrotic region” and “calcified necrosis.”7,8 These assumptions are also reflected in the published VH IVUS tissue component classification tree, where most of the necrotic core leaves are in the high-intensity branches.7 This is counterintuitive when taking the conventional wisdom related to gray-scale IVUS into account.

Looking at the gray-scale IVUS in Figure 2, it would be reasonable to suspect a necrotic core in the echolucent area at the 9 o’clock position, which is in agreement with the histological findings. Looking at the gray-scale IVUS in Figure 3, one would probably not have suspected a necrotic core, which is in agreement with the histological findings. VH IVUS red areas do not correspond to gray-scale IVUS echolucent areas, nor do VH IVUS red areas correspond to necrotic core location by histology. This supports conven-
tional wisdom related to gray-scale IVUS tissue characterization capabilities and seriously questions that VH IVUS has incremental value in terms of tissue characterization when added to gray-scale IVUS investigation.

Nomenclature for Necrosis in VH IVUS and the Calcium Challenge

Today, VH IVUS reports red for “necrotic core.” Earlier, however, the red area was referred to as “calcified necrotic regions” or “calcified necrosis,” reflecting that calcification was included in the algorithm for the detection of “necrotic core.” In fact, when the VH IVUS tissue component classification tree was first published, calcified necrotic regions and calcified regions could not be separated on the basis of spectral parameters and were combined in the analysis. Later, accuracies in component detection were still improved when these 2 components were combined.

Our data suggest that the coupling between calcium and necrosis combined with inadequate ability to distinguish calcium from fibrosis may pose a problem. As illustrated in Figure 3, very dense fibrous tissue can be interpreted as necrotic core by VH IVUS. A stable fibrous plaque without necrosis therefore could be falsely labeled as vulnerable. This inability to distinguish between collagen and calcified necrosis has been noted earlier. Also, the published classification tree revealed that calcified, calcified necrotic, and some fibrous regions are in the same cluster without significant differences in spectral parameters between calcified necrotic and fibrous regions.

Necrosis Versus Non-Necrotic Areas With High Lipid Content

Our data also suggest that VH IVUS may have difficulties accurately discerning necrotic core and fibrofatty regions (non-necrotic areas with high lipid content) when calcifications are not present in the necrotic core or present but missed by VH IVUS. In Figure 2, a large necrotic core was missed and classified fibrofatty by VH IVUS (around the 9 o’clock position). This may illustrate the untoward effect of setting calcification as a prerequisite for necrotic core. This has also been acknowledged and commented on earlier.

Figure 4. Effects of changing the vessel border on tissue characterization by VH IVUS. The interrogated lesion is that shown in Figure 3. A through C, VH IVUS display with 4 color codes: red for necrotic core, light green for fibrofatty tissue, dark green for fibrous tissue, and white for dense calcium. A through C show the same VH IVUS frame with the lumen border held constant while the vessel border is moved. A, Vessel border moved inward. B, Vessel border as shown in Figure 3. C, Vessel border moved outward. The VH IVUS composition analysis of the innermost and critical part of the plaque did not change by changing the vessel border. Regardless of the vessel border location, VH IVUS reported necrotic cores and calcifications in a fibrous and rather homogeneous neointimal lesion without necrotic cores or calcifications by real histology (Figure 3).

Figure 5. Effects of changing the luminal border on tissue characterization by VH IVUS. The interrogated lesion is that shown in Figure 3. A and B, VH IVUS display with 4 color codes: red for necrotic core, light green for fibrofatty tissue, dark green for fibrous tissue, and white for dense calcium. A and B show the same VH IVUS frame with the vessel border held constant while the luminal border is moved. A, Lumen border as shown in Figure 3. B, Lumen border moved into the plaque middle. The VH IVUS composition analysis of the innermost and critical part of the plaque changed when the luminal border was moved. The composition analysis of the deeper parts of the plaque did not change. Regardless of the luminal border location, VH IVUS reported necrotic cores and calcifications in a fibrous and rather homogeneous neointimal lesion without necrotic cores or calcifications by real histology (Figure 3).
Detection of TCFA With VH IVUS

The fibrous cap is, by definition, the fibrous tissue separating the necrotic core from the lumen.\(^27\) If the necrotic core is ill defined by VH IVUS, so is the fibrous cap, which leaves reliable detection of fibrous cap and discrimination of a thin fibrous cap from a thick one with this technology questionable, not only because of limited spatial resolution.\(^38\)

Study Limitations

Aligning IVUS frames and histology slides is challenging and may provide some inaccuracy. Also, VH IVUS was not developed for porcine lesions, which may differ in composition from human lesions, although the present porcine coronary artery disease model bears large resemblance to human coronary disease. Additionally, we tested VH IVUS on balloon-accelerated lesions, although VH IVUS was developed on native lesions. However, VH IVUS is applied to a wide range of lesion types in clinical research and practice.\(^29,30\) For the interpretation of such studies, experimental testing of VH IVUS on a wider range of lesions is necessary.\(^31\) Notably, analyzing spontaneously developed lesions separately, necrotic core size evaluated with VH IVUS and histology did still not correlate.

Conclusion

Although widely applied, VH IVUS has never been validated for quantification of plaque components. We found no correlation between necrotic core size defined by VH IVUS and histology. This may, at least in part, be explained by the fact that the VH IVUS algorithm relies on the presence of calcifications for necrotic core detection. Our study warrants caution in the interpretation of studies relying on VH IVUS for monitoring of treatment effects, event prediction, and validation of other imaging modalities.

Acknowledgments

We thank Birgitte Kildevæld Sahl, Rita Ullerup, and Lisa Maria Røge for their expert technical assistance in preparation of the histology.

Sources of Funding

The study was supported by Aarhus University Research Foundation, The Danish Medical Research Council, and The Danish Cardiovascular Research Academy (DaCRA).

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Intravascular ultrasound–derived virtual histology (VH IVUS) is currently used extensively in clinical research, and necrotic core area assessed with VH IVUS is used as a primary end point in clinical trials. VH IVUS has also been proposed as a potential vulnerable plaque detector, based on its capability to assess the area of the necrotic core of thin-cap fibroatheromas. However, VH IVUS has never been validated for assessment of necrotic core area. In this animal study, we found that VH IVUS did not reliably assess necrotic core area in porcine coronary artery disease. This finding raises concern about using necrotic core area, as assessed by VH IVUS, as an end point in clinical trials. It should also alert clinicians using VH IVUS in the catheterization laboratory about the potential pitfalls of using this to identify vulnerable plaque until more fully validated.
Unreliable Assessment of Necrotic Core by Virtual Histology Intravascular Ultrasound in Porcine Coronary Artery Disease
Troels Thim, Mette Kallestrup Hagensen, David Wallace-Bradley, Juan F. Granada, Greg L. Kaluza, Ludovic Drouet, William P. Paaske, Hans Erik Bøtker and Erling Falk

Circ Cardiovasc Imaging. 2010;3:384-391; originally published online May 11, 2010;
doi: 10.1161/CIRCIMAGING.109.919357
Circulation: Cardiovascular Imaging is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-9651. Online ISSN: 1942-0080

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circimaging.ahajournals.org/content/3/4/384

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Cardiovascular Imaging can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Cardiovascular Imaging is online at:
http://circimaging.ahajournals.org//subscriptions/