Plaque Neovascularization is Increased in Human Carotid Atherosclerosis Related to Prior Neck Radiotherapy

A Contrast Enhanced Ultrasound Study

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ABSTRACT

Objective

To determine the effect of radiotherapy (RT) on intra-plaque neovascularization (IPN) in human carotid arteries.

Background

Exposure of the carotid arteries to RT, during treatment for head and neck cancer (HNC), is associated with increased risk of stroke. However, the effects of RT upon IPN – a pre-cursor to intraplaque hemorrhage and thus associated with plaque vulnerability – are unknown.

Methods

Patients that had undergone unilateral RT for HNC $\geq$ 2 years previously underwent B-mode and contrast-enhanced carotid ultrasonography (CEUS) of both RT-side and non-RT side arteries. Presence of IPN during CEUS was judged semi-quantitatively as grade 0 (absent), grade 1 (present but limited to plaque base) or grade 2 (extensive and noted within plaque body).

Results

Of 49 patients studied, 38 had plaques. The number of plaques were significantly greater in the RT versus the non-RT arteries. Overall, 75% RT-side plaques had IPN vs. 39% non-RT side plaques (p=0.002). Amongst patients with plaques, IPN was present in 81% RT-side plaques vs. 41% non-RT side plaques (p=0.004). Grade 0 IPN was significantly more common in non-RT side plaques (25% vs. 61%, p=0.002) whereas Grade 2 plaques were more common on the RT side (31% vs. 9%, p=0.03). The only clinical variable which predicted presence or absence

of IPN was RT laterality.

Conclusion

This is the first study in humans to reveal that there is a significant association between RT and presence and extent of IPN. This may help to explain the etiology of increased stroke risk amongst HNC survivors treated by RT.

Key words

Plaque neovascularization; contrast; contrast-enhanced ultrasound; radiotherapy

INTRODUCTION

Atherosclerosis is a chronic inflammatory condition and the underlying patho-biological substrate that accounts for the majority of cardiovascular events. However, not all patients with atherosclerosis experience such outcomes. Over the past two decades, much research interest has focused upon predicting which atherosclerotic plaques will 'rupture' – leading to events such as myocardial infarction or stroke – and which plaques do not rupture, leading to the concept of the 'vulnerable' (or unstable) plaque.(1)

An emerging key feature of such plaques are intra-plaque neovessels. These neovessels are fragile, leaky and prone to rupture, leading to intra-plaque hemorrhage, which contributes to the necrotic core of plaques and is believed to increase risk of plaque rupture.(2) Consequently, the presence of intra-plaque neovascularization (IPN) is widely thought of as a pre-cursor to the vulnerable plaque.(3,4) A number of imaging and histological observational studies have revealed a clear association between presence and extent of IPN and subsequent cardiovascular events, including mortality.(5-7) Much of this research in humans has focused upon the carotid arteries, firstly due to their superficial location (favours non-invasive imaging techniques), secondly because of the association with stroke and thirdly because patients undergoing carotid endarterectomy provide a suitable model for histological comparisons.

IPN can be visualized by several non-invasive imaging techniques, one of which is contrast-enhanced ultrasound (CEUS). CEUS utilizes manufactured trans-pulmonary ultrasound contrast agents, which consist of acoustically-active gas-filled microbubbles. These microbubbles, which remain intravascular at all times, are typically slightly smaller than erythrocytes, allowing free passage within the circulation and effectively acting as red cell "tracers". Studies have demonstrated that carotid ultrasonography performed following

administration of contrast permits visualization of IPN (8) and comparisons with histological neovessel density have validated the technique's accuracy.(9)

Radiotherapy (RT) damages arterial walls and promotes atherosclerotic plaque formation. The carotid arteries frequently receive significant incidental doses of radiation during RT treatment of malignancies, especially head and neck cancers (HNC). Radiation vasculopathy had until recently been considered quite a rare entity, as patients would frequently succumb to their malignancy first. However, as cancer survival rates improve, patients are "outliving" their malignancies and presenting later with the long-term sequelae of cancer therapy.(10)

Several studies have shown that RT of the carotid arteries is associated with increased intima-media thickness (IMT), increased carotid plaque formation and thus overall an increased risk of stroke.(11) However, although the effects of RT on carotid IMT and plaque burden have been documented, the effect of RT on plaque composition – specifically IPN – has not been studied in humans. We thus performed this cross-sectional study to assess the effects of RT upon IPN in survivors of HNC that had previously received RT.

METHODS

Study Design

This was a cross-sectional study of patients previously treated at a cancer center with RT for HNC. Ethical approval for this study was obtained and all patients provided informed written consent. The inclusion criteria were as follows:

- Age >18 years

- Histologically confirmed cancer treated with hemi-neck RT to ≥ 50Gray (Gy)
- Radiotherapy administered > 24 months previously
- Able to provide written informed consent

The exclusion criteria were as follows:

- Patients with active HNC
- Patients with a prior history of carotid endarterectomy or carotid angioplasty / stenting
- Patients with bilateral RT
- Known allergy to sulphur or sulphur-containing drugs

HNC patients who had received unilateral radical RT prior to December 2009 were identified via the RT database. Eligible patients were contacted and those agreeable to participation (only three patients declined) in the study were then invited to attend for a baseline questionnaire, brief physical examination, routine blood tests (including full blood count and biochemistry and total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels) and carotid ultrasonography.

Patient Clinical Variables

Presence of cardiovascular risk factors were defined as follows: diabetes mellitus (random serum glucose ≥ 11.1mmol/L, a glycosylate haemoglobin A1c (HbA1c) ≥5.8% or current use of glucose-lowering agents or insulin), hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or current use of ant-hypertensive agents), hyperlipidemia (fasting serum low density lipoprotein (LDL) ≥2.6mmol/L, high density lipoprotein (HDL) <2.3mmol/L or triglycerides (TG) ≥2.3mmol/L, or current use of

cholesterol-lowering agents), ever smoker and family history of premature CAD (first degree relative suffered myocardial infarction or stroke, <55yrs (men) or <65yrs (women)). Height (in metres) and weight (in kilograms) were recorded in order to calculate body mass index (BMI).

Carotid Ultrasonography

B-mode, colour Doppler and contrast enhanced carotid ultrasonography were performed using a high-resolution ultrasound system (Vivid-7; General Electric Healthcare, Chalfont, Bucks, UK) equipped with a broadband linear array (3-11 MHz) transducer, as illustrated in figure 1. All scans were performed by cardiologists (BNS & NSC) blinded to the patient's prior history including the laterality of RT. ECG monitoring was performed continuously throughout the scan and arterial blood pressure was recorded using an automated sphygmomanometer. In summary, the proximal, mid, and distal common carotid artery (CCA), bifurcation of the CCA and proximal portion of the internal and external carotid arteries were systematically interrogated in long-axis and short-axis views. Color Doppler imaging was used to identify flow and spectral Doppler used to measure flow velocities.

Plaque was defined as per the Mannheim consensus as a focal structure encroaching into the arterial lumen by >0.5 mm, a distinct area of IMT >50% greater than the adjacent wall or >1.5 mm in thickness.(12) IMT measurements were taken at the far wall of the distal CCA at end-diastole using a semi-automated edge detection algorithm (EchoPAC version 8.0, GE Healthcare) and the mean value obtained was an average of 3 measurements.

After B-mode images were acquired, an intravenous cannula was inserted and Sonovue ultrasound contrast (Bracco Diagnostics, Milan, Italy) was administered as a continuous intravenous infusion using a specific pump (Vueject, Bracco, Milan, Italy). Two vials of

Sonovue (total 8mL) were infused at a standardized rate of 1.2ml/min, providing approximately 6.5 minutes of contrast opacification. During this time, the right and left carotid arteries were re-imaged, with special focus upon areas of abnormality (i.e. plaques) identified during the B-mode scan. As previously described in a CEUS study with histological validation (9), IPN was graded semi-quantitatively as absent (Grade 0), limited to the adventitia / plaque base (Grade 1) or extensive and/or extending into the plaque body (Grade 2) by a doctor blinded to the side of RT. Following the scan, all images were transferred to the EchoPAC database and also stored on disc for off-line analysis.

Statistics

The primary aim of the study was to compare carotid plaques in irradiated arteries to unirradiated internal controls. As previously reported, the expected absolute difference in the prevalence of carotid artery stenosis due to plaque is around 25-30% (13-15). With a Type I error set at 95% ($\alpha = 0.05$) and a type II error set at 10% ($\beta = 0.10$), a minimum of 80 patients would be required. As patients treated with unilateral RT were evaluated (the contralateral carotid artery served as an internal control), a total of 40 patients were therefore required for this study. In order to account for unexpected loss of subjects (e.g. poor ultrasound windows, withdrawal of consent), the study aimed to recruit a total of 50 patients. As an increased prevalence of carotid plaque was expected on the RT-side, we hypothesized that there may also be an accompanying increased incidence of IPN also.

Continuous variables are presented as mean ± standard deviation and categorical variables as proportions. Continuous variables were compared using the Student's t-test and categorical variables using Chi-square test. Intra-observer and inter-observer variability were tested for using kappa. All statistical calculations were performed using SPSS version 19.0. A p value

<0.05 was taken as statistically significant for all tests.

RESULTS

Patient Variables

A total of 50 patients consented for the study, of whom 49 underwent B-mode and contrast-enhanced carotid ultrasound studies (in one patient it was not possible to obtain intravenous access and thus contrast could not be administered). The baseline demographics of these 49 patients are detailed in Table 1. Mean age was 57±8yrs, 69% were male and mean BMI was 26.3±4.4 kg/m^2. The histological tumour type was squamous cell carcinoma in the majority of patients (38/49, 78%).

Almost half the cohort (22/49, 45%) had also received platinum-based chemotherapy drugs for treatment of their HNC. The mean time duration from RT to carotid imaging was 5.4±2.5 years (range 2.1 – 12.6yrs). Routine full blood count was normal in all patients. Mean serum creatinine value was 75±19mmol/L (range 46-128mmol/L). The mean total, HDL and LDL cholesterol levels were 5.3mmol/L, 1.5mmol/L and 3.2mmol/L respectively. All patients had normal serum calcium concentration (mean 2.2±0.1mmol/L).

Carotid Ultrasonography

The data presented in Table 2 demonstrates the differences in plaque number, area and prevalence of IPN from the RT and non-RT carotid arteries. Of the 49 patients examined, plaques were detected in 38 (78%). There were a greater number of plaques on the RT side although mean plaque area and total plaque burden by area were similar between RT and non-RT arteries.

IPN was analyzed on a per-patient as well as per-plaque basis, but in both cases, IPN was more commonly seen on the RT side than the non-RT side. IPN was visualized in 75% plaques from the RT-side artery vs. just 39% from non-RT arteries. The absence of IPN (i.e. Grade 0) was significantly more frequent in non-RT plaques than RT plaques. Conversely, Grade 2 IPN – the highest grade and indicative of the most extensive IPN – was significantly more commonly identified in RT plaques than in non-RT plaques. Figures 2 shows a patient with a large plaque but without IPN (i.e. Grace 0) whereas figures 3 and 4 illustrate patients with IPN clearly seen during CEUS imaging.

We examined the impact of patient variables on presence or absence of IPN, both in all patients and the patients with IPN on the RT side plaques only and these results are shown in table 3. In summary, there was no statistical relationship between presence or absence of IPN and gender, smoking history, body weight, prior treatment with chemotherapy, time duration since RT or prior use of aspirin. Patients taking statins were less likely to have IPN than patients not on statin therapy, though this had borderline statistical significance ($p=0.053$). Inter-observer variability for both presence / absence of IPN and for grade of IPN had a kappa value of 1.0.

DISCUSSION

This is the first study to assess the impact of RT upon plaque neovascularization. Our results have shown that IPN is significantly increased in plaques from arteries exposed to RT compared to arteries that did not receive RT. Irrespective of whether our data were analyzed on a per-patient or per-plaque basis, IPN was more common in RT-side plaques and extensive IPN – Grade 2 – was also significantly more frequent on the RT side than non-RT side. These findings were independent of age, gender, smoking status, time duration since RT, drugs

(including aspirin, statins and chemotherapy) and BMI. Indeed, the only variable associated with presence of IPN was RT laterality.

The pathophysiology of radiation vasculopathy is poorly understood, in large part due to the lack of a definitive clinicopathologic study in humans. Three mechanisms have been proposed – ischemic necrosis (due to occlusion of the vasa vasorum), adventitial fibrosis (leading to external arterial compression) and accelerated atherosclerosis.(16) Animal studies have given an insight into the changes seen in arterial walls with time following RT, progressing from initial endothelial damage and then thickening to adventitial fibrosis and necrosis of the media also.(17)

However, it is widely believed that the initial injury to the vasa vasorum is a key feature of RT-related arterial disease, though it has remained unclear whether this process is predominantly inflammatory or ischemic in nature (ischemia secondary to microthrombosis / occlusion of the vasa vasorum).(18) However, our results show that the vasa vasorum have proliferated markedly into the plaques (IPN), rather than being reduced, favoring an inflammatory process. In 'conventional' (i.e. non-RT related) atherosclerosis, proliferation of the adventitial vasa vasorum is triggered by increased production of hypoxia inducible factor (HIF), a response to reduced local oxygen tension due to increased thickness of the intima-media complex. As RT is known to cause increased IMT – as we also observed in this study – it is not surprising that a greater degree of IPN was observed on the RT side.

Limitations of Current Evidence

Radiation vasculopathy has been studied previously and many groups have investigated the effects of head and/or neck RT upon carotid arteries (imaging studies) and future CVE

(outcome studies), which have recently been comprehensively reviewed.(19) However, for a multitude of reasons, the literature displays marked heterogeneity in this field with a number of problems associated with these studies. First, studies that have reported an increased relative risk of CVE after RT have either used non-matched control groups or have matched their HNC patients to geographically distinct and distant population data(20,21), sometimes derived from another country. Studies have not always reported upon stroke sub-type (ischemic or hemorrhagic) (22) and information on CVE has not always been judged by a clinician or by brain imaging, but by patient questionnaires.(23) Additionally, though many studies have suggested an increased risk of CVE in patients exposed to head/neck RT, at least two studies – which did have well-matched control groups – did not find an increased risk of CVE on long-term follow-up.(24,25) Finally, and possibly most importantly, the majority of these studies did not provide data on the laterality of the stroke. For example, if the right carotid artery has been exposed to RT, a clinically ischemic event would be expected in the right cerebral hemisphere and thus produce left sided signs (e.g. paresis, reduced sensation). However, this level of detail is not available in many prior research papers (20,22,23); thus, although one would assume that an ischemic CVE must be related to the RT-side carotid artery, the data confirming this are absent.

As a result of these short-comings in the existing data, several questions regarding radiation vasculopathy remain answered, most prominent of which is the mechanism by which RT affects the arterial wall and subsequently appears to increase stroke risk. The increased frequency of IPN we observed strongly implicates an inflammatory reaction, in keeping with conventional models of atherosclerosis. Indeed, IPN is one of several initial defence mechanisms in response to atherosclerosis.(26) Of the three layers of the arterial wall, the tunica media and adventitia receive blood supply from the vasa vasorum, whereas the tunica

intima is dependent upon diffusion of oxygen directly from the lumen. With development of an atherosclerotic plaque within the intima, the distance between the deeper intimal layers and the luminal surface increases, producing hypoxia within the plaque. This stimulates release of pro-angiogenic factors that induces proliferation of the adventitial vasa vasorum and growth of neo-vessels in an attempt to restore normal oxygen tension. Eventually, the plaque is enveloped in extensive vasa vasorum and IPN, a hallmark of atherosclerosis.

Clinical Implications

There is increasing evidence that plaque composition is clinically important – independent of stenosis severity – in relation to outcome.(27,28) As a result of large international surgical trials, carotid endarterectomy is reserved for patients with symptomatic arterial plaques with stenosis severity >70-80% as judged by Doppler ultrasound.(29,30) However, it is not known whether superior risk stratification could be achieved by accounting for novel markers of plaque vulnerability, such as presence or absence of IPN, rather than just lesion severity alone.

Our results demonstrate that patients with RT-related carotid disease have a significant increase in IPN, a marker of plaque instability. One would therefore hypothesize that these patients with increased IPN are at increased risk of CVE. It is unknown whether surgical removal of these plaques, even if only causing mild or moderate stenosis, is preferable to medical therapy alone. However, a randomized controlled trial examining this very question appears justified on the basis of our findings.

Strengths and Limitations

Our study has certain strengths and limitations that warrant discussion. Strengths include a well-defined patient population, blinding of the carotid scanner from side of RT treatment, use

of the contra-lateral artery as an ‘internal’ self-control and, uniquely, use of a continuous intravenous infusion of contrast which, unlike multiple bolus injections, produces a constant concentration of microbubbles within the bloodstream.

Limitations include lack of another imaging modality for comparison and the lack of histology for verification. However, regarding imaging, CEUS has been shown as an accurate technique and carotid MRI, for example, has not been shown to be superior for identification of IPN. It is true that carotid MRI can assess more features of a plaque’s composition than CEUS, including size of the lipid core and thickness of the fibrous cap. However, these aspects of plaque composition were not the primary focus of this research project. Regarding histology, these patients had no clinical indication for carotid surgery thus histology was impossible to obtain, though previous studies have verified the accuracy of CEUS for accurate identification of presence and extent of IPN.

Conclusions

Plaque neovascularization, a pre-cursor to plaque hemorrhage and thus a surrogate for plaque instability, is significantly increased in arteries exposed to RT during treatment of HNC. This effect of RT upon IPN is independent of all other clinical variables, including age, gender and previous treatment with chemotherapy. These results suggest that the atherosclerotic plaques of radiation vasculopathy may demonstrate increased vulnerability and this may help to explain the greater risk of CVE in this patient population. Long-term follow-up of this patient cohort will identify the prognostic impact of IPN in this patient population.

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FIGURES

Figure 1: Examples of B-mode (left), colour Doppler (center) and CEUS (right) imaging of the carotid arterial tree

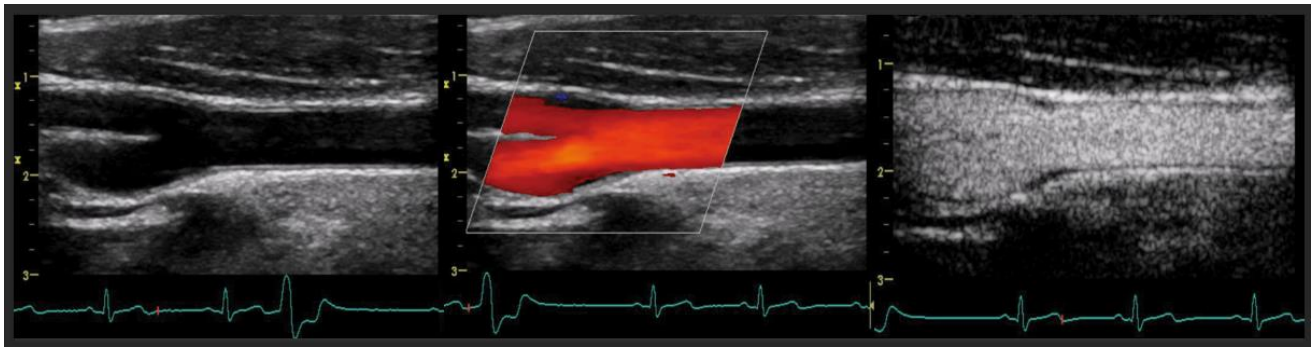


Figure 2: Long axis example of a large far wall plaque with no IPN detected during CEUS (also see Supplementary Movie 1)

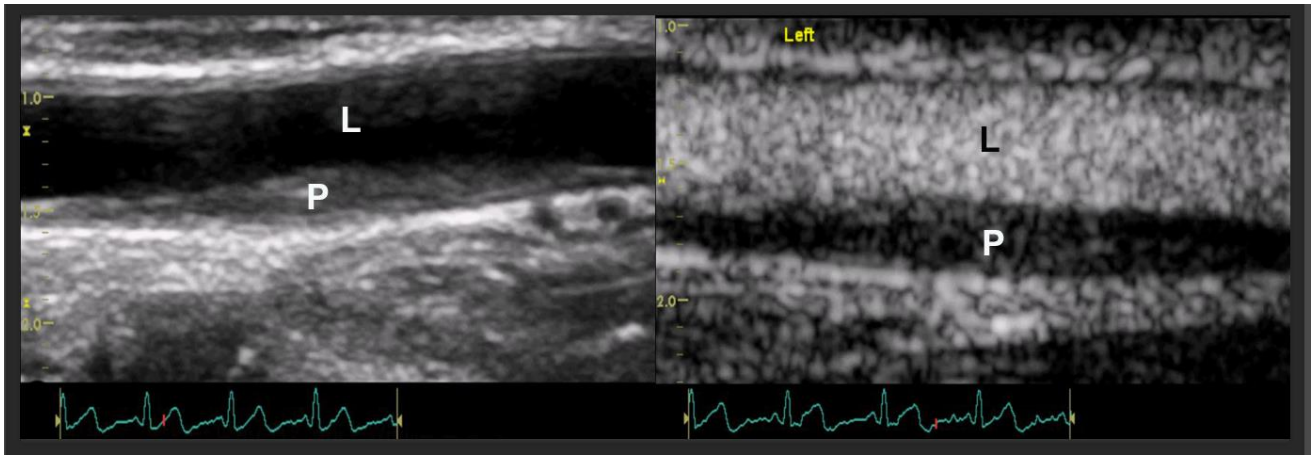


Figure 3: Long axis example of a large plaque with IPN seen (arrows) during CEUS (CCA = Common carotid artery, ICA = Internal Carotid Artery, ECA =External Carotid Artery – also see Supplementary Movie 2)

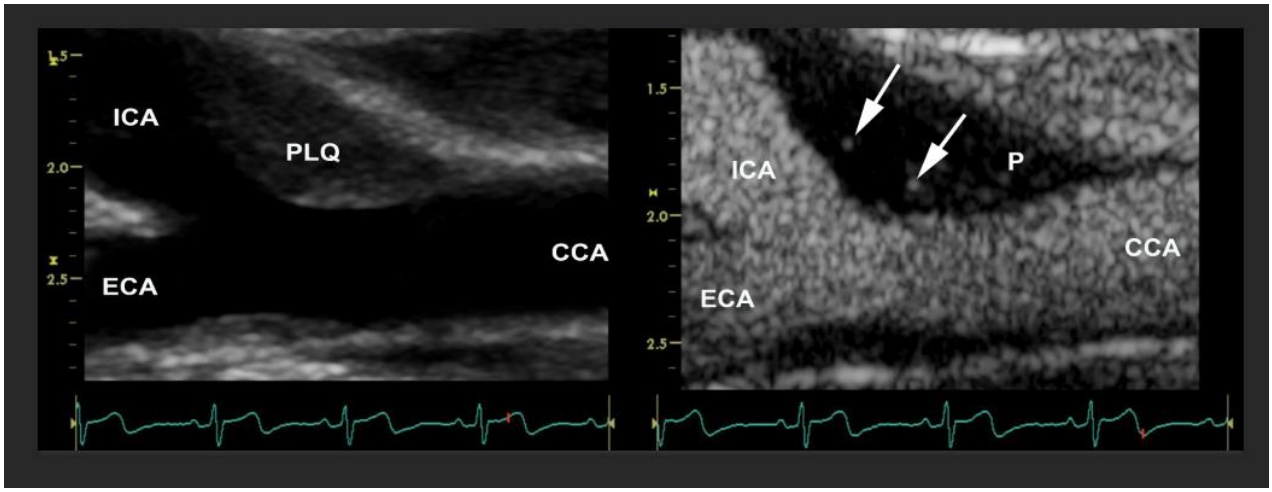
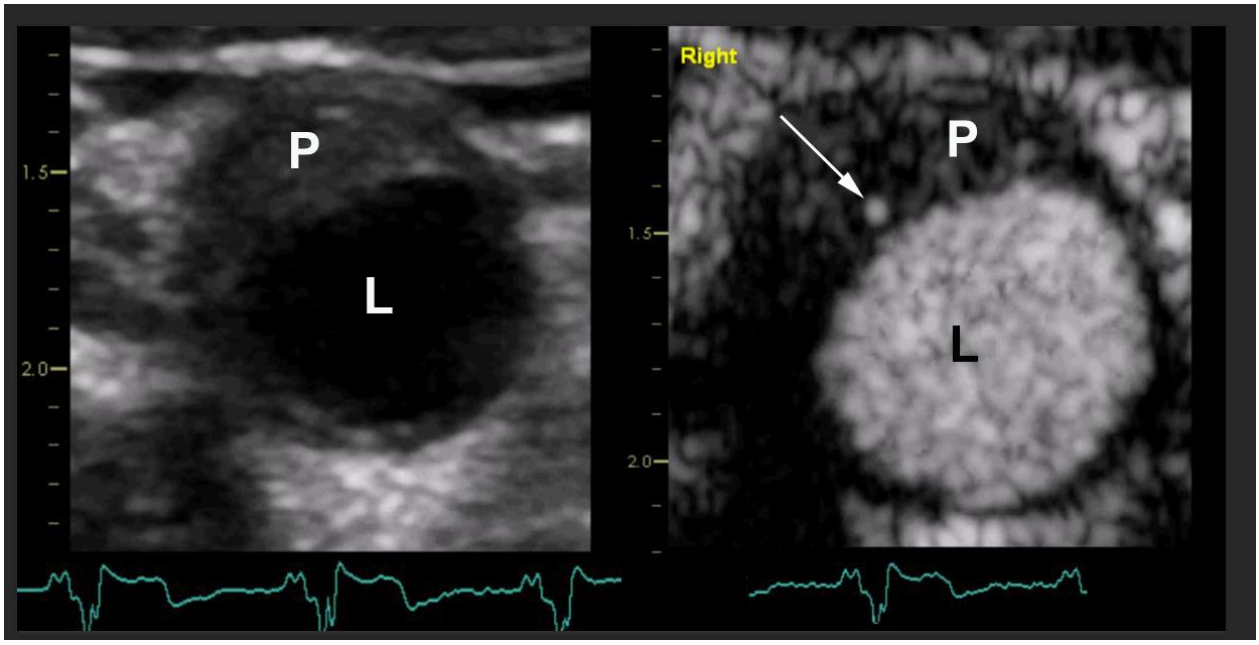


Figure 4: Short axis example of a large plaque (P), causing significant reduction in lumen (L) diameter, with clear IPN visualized during CEUS (also see Supplementary Movie 3)



TABLES

Table 1: Baseline patient characteristics

Patient characteristics	**Number (%)** (n = 49)
Demographics	
Mean age (yrs±SD)	57±8
Male gender	34 (69.0)
Diabetes	4 (8.2)
Hypertension	13 (26.5)
Smoker	26 (53)
Hyperlipidemia	11 (22.4)
Mean BMI (±SD)	26.3±4.4
Mean time from RT (years)	5.4±2.5
Medications	
Anti-hypertensive drugs	13 (26.5)
Anti-diabetic drugs	4 (8.2)
HMG Co-A reductase inhibitors (Statins)	11 (22.4)
Aspirin	13 (26.5)
Loop or thiazide diuretic	5 (10.2)

Table 2: Results of B-mode and CEUS imaging in RT-side and non-RT side arteries

VARIABLE	RT SIDE n (%)	NON-RT SIDE n (%)	P VALUE
Mean Intima-Media Thickness (mm)	0.77±0.20	0.68±0.16	**0.02**
Total Number of Plaques	64/87 (74)	23/87 (26)	?
Mean Plaque Area (mm^2)	23.1±13.8	21.0±14.5	0.57
Mean Total Plaque Area (mm^2)	39.1	28.5	0.25
Per-Patient Basis			
Plaque present?	36/49 (73)	17/49 (35)	**<0.001**
IPN detected?	29/36 (81)	7/17 (41)	**0.004**
Grade 2 IPN detected?	14/36 (39)	2/17 (12)	**0.04**
Per-Plaque Basis			
No. of plaques with IPN detected	48/64 (75)	9/23 (39)	**0.002**
No. of plaques with IPN category:			
Grade 0	16/64 (25)	14/23 (61)	**0.002**
Grade 1	28/64 (44)	7/23 (30)	0.26
Grade 2	20/64 (31)	2/23 (9)	**0.03**

Table 3: Relationship between presence or absence of IPN and patient variables in all patients (n=49, left) and RT-side only plaques (n=36, right)

	ALL PATIENTS			RT-SIDE ONLY		
Variable	**IPN N=30 (%)**	**No IPN N=19 (%)**	**p value**	**IPN N=29 (%)**	**No IPN N=7 (%)**	**p value**
Male Gender	23 (77)	11 (58)	0.17	22 (76)	4 (57)	0.58
Smoking	18 (60)	8 (42)	0.22	18 (62)	2 (29)	0.13
Aspirin use	9 (30)	4 (21)	0.49	8 (28)	2 (29)	0.95
Statin use	7 (23)	4 (21)	0.85	6 (21)	4 (57)	0.05
BMI >25kg/m^2	17 (57)	6 (32)	0.80	19 (66)	5 (71)	0.77
RT >5yrs	17 (57)	6 (32)	0.09	17 (59)	3 (33)	0.15
Chemotherapy	13 (43)	9 (47)	0.78	12 (41)	2 (29)	0.53