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Wall shear stress and local plaque development in stenosedcarotid arteries of hypercholesterolemic minipigs

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ABSTRACT

Background: It is believed that wall shear stress has a significant impact on plaque susceptibility and the local development of atherosclerotic plaque. The relationship between wall shear stress and local plaque development is not entirely explained by the models and theories in use today, though. Our goal was to look at the relationship between local plaque development and wall shear stress in surgically created common carotid artery stenoses in minipigs with high cholesterol. Supplies, Procedures, and Outcomes: Using a perivascular collar, we surgically induced stenosis of the common carotid artery in ten minipigs. Using computational fluid dynamics, magnetic resonance imaging, and ultrasound, we recorded the variations in flow and shear stress. Microscopy was used to document carotid plaques. Thrombus was linked to atherosclerotic lesions in both the pre- and post-stenotic portions of the

Key words: Atherosclerosis, carotid artery, magnetic resonance imaging, vulnerable plaque, wall shear stress

INTRODUCTION

Vulnerable atherosclerotic plaques with a necrotic core may rupture and cause thrombosis.[1] This is the main mechanism underlying coronary and carotid thrombosis.[2-4] Low or oscillatory wall shear stress is thought to play a critical role in the development of atherosclerosis and may also affect plaque vulnerability.[5,6] In experimental models, In order to alter wall shear stress, common carotid blood flow is disrupted through surgically induced constriction of the artery. This can cause atherosclerotic plaques in hypercholesterolemic mice and pigs that resemble human susceptible plaques in the common carotid artery, which is otherwise spared from atherosclerosis, even in animals with elevated cholesterol levels.[6–13]

The murine carotid stenosis model has been used to draw findings in the past regarding the effects of low and oscillatory shear stress.[6] Based on anatomical theory and a Doppler ultrasonography blood flow measurement, wall shear stress was calculated. It was determined that lesions with a susceptible phenotype formed when low shear stress occurred close to the stenosis, and that oscillatory shear stress occurred when low shear stress occurred farther away from the stenosis.

keeping a constant phenotype. This does not explain why the femoral artery is less prone to atherosclerosis than the left anterior descending artery or the carotid artery, even though these arteries have lower wall shear stress. Neither does it explain why plaque typically develops immediately proximal to the stenosis and not in the entire pre-stenotic segment, which has the same low flow and shear stress.[16] Post-stenotic dilatation is also linked to severe stenosis. Post-stenotic dilatation should be linked to a lower linear flow velocity and, consequently, wall shear stress distal to the stenosis compared to proximal to the stenosis, given the same volumetric flow both proximal and distal to the stenosis.

To address some of these unresolved questions, we aimed to investigate the relation between wall shear stress and local plaque development in surgically induced common carotid artery stenoses of hypercholesterolemic minipigs. Minipigs are closer to human size and thereby a more detailed assessment of carotid flow and anatomy is possible. We assessed carotid artery blood flow and anatomy with magnetic resonance imaging and performed a detailed description of local wall shear stress with computational fluid dynamics. Local development of atherosclerotic lesions was assessed by microscopic examination.

MATERIALS AND METHODS

This study was approved by the Danish Animal ExperimentsInspectorate.

Minipigs and protocol

Ten male castrated minipigs, with familial hypercholesterolemia due to a low-density lipoprotein receptor mutation,[17-19] were fed an atherogenic diet for 18 weeks. After 4 weeks on the atherogenic diet, surgical stenosis of one carotid artery was induced by placement of a silicone collar, with an inner diameter of 2 mm, around the carotid artery. The intention was to create common carotid artery stenoses that reduced the common carotidartery blood flow by appro[imately 75%. The collar was made from a silicone tube slit open longitudinally. Thisallowed its placement around the artery. In place, the tube was closed with two ligatures to form the stenosingcollar. The collar then divided the common carotid artery into three segments: A pre-stenotic segment, a stenosedsegment, and a post-stenotic segment [Figure 1]. Carotid blood flow was measured perioperatively in the pro[imalsegment before and after the placement of the collar with a transit time flow probe.[20] To reduce the risk of clotting and thrombosis, the minipigs received unfractioned heparinin relation to the procedures and aspirin (150 mg daily) after the procedures.

During the study, the minipigs were weighed and bloodsamples were drawn after 2 weeks, 4 weeks, 14 weeks and18 weeks on the atherogenic diet and analyzed for plasmacholesterol.

Carotid ultrasound

Twelve weeks following surgery, carotid ultrasonography was carried out on minipigs that were anaesthetized with azaperone and midazolam. The GE LOGIQ 9, GE Healthcare, Brøndby, Denmark equipment was used to conduct colour and spectral Doppler investigations. Depending on the depth, a 4 MHz multifrequency curved array or a 10 MHz multifrequency linear transducer was used. The proximal and stenosed regions underwent two separate acquisitions of spectral Doppler data. The ultrasound device's software used the artery diameter and mean flow velocity to calculate the flow volume. The two measures' mean value was employed. The peak flow velocity ratio between the proximal and stenosed segments was used to calculate the degree of stenosis.[21] Among the two readings, the highest peak flow value was employed.

MRI and wall shear stress assessment

MRI was performed on seven of the minipigs immediatelybefore termination of the study. During the scan, the minipigs were sedated with a continuous propofol infusion.From stacks of 45 MR images of each carotid artery, manualsegmentations were performed using ScanIP (SimplewareLtd., Exeter, UK) generating three-dimensional models ofthe carotid arteries. The models were further processed

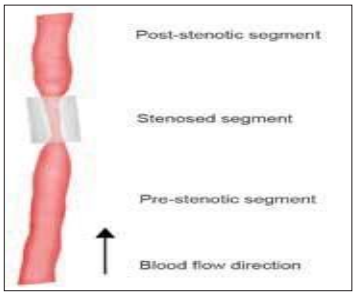

Figure 1: Common carotid artery collar model. Surgical stenosiswas induced by placement of a silicone collar (inner diameter 2 mm, length 1 cm) around the carotid artery. The collar divided the common carotid artery into three segments: A pre-stenotic segment, a stenosedsegment, and post-stenotic segmentmaking a mesh for computational fluid dynamics using ScanFE (Simpleware). The Finite Element programme Comsol Multiphysics 3.4 (COMSOL inc., Stockholm, Sweden) was opened with the mesh imported. The inlet boundary conditions were applied to the models in Comsol Multiphysics as time-dependent velocities. The average cross-sectional velocity of the carotid artery was measured at 20 different time steps during a heart phase using the velocity data that were acquired from the MRI phase contrast flow measurement. A pressure condition with a value of 0 served as the outflow boundary condition.

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Blood was simulated as being a homogenous Newtonianfluid with a density of 1030 kg/m and a viscosity of $3.6 \cdot 10^{-3}$Pa·s. In all simulations, velocities at the wall boundaries were set to zero, the so-called "no slip" condition.Arterial compliance was considered negligible and was notincluded. Numerical time dependent simulations of blood flow within the carotid arteries were performed employing the generalized isothermal Navier-Stokes equation formulated as conservation of mass and momentum in Comsol Multiphysics. Wall shear stress data were derivedfrom time-dependent simulations by calculating the viscousstress tensor c and visualized using the postprocessing tools in Comsol. In addition, visualization of the blood flow streamlines were also performed using the ComsolInterface. From the time-dependent simulations, wall shearstress data were derived for each time step in the simulation.These data were further processed using Matlab R2007b(MathWorks, Natick, Massachusetts, U.S.) to visualize thelocation and magnitude of oscillatory wall shear stress.

Angiography

Conventional angiography was performed immediatelybefore termination. Stenosis length and luminal diameters in the proximal, stenosed and distal segments, and angiographic stenosis degree ([1-stenosis diameter/ proximal diameter]·100) and distal dilatation (distal diameter/proximal diameter-1) was calculated.

Microscopic examination

The carotid arteries were e[cised and pressure fi[ed with4% formaldehyde at 100 mmHg for 1 h and subsequently immersion fi[ed for 6–12 h. The arteries were sectioned at 4-mm intervals, paraffin embedded, and sectioned formicroscopic examination. Hematoxylin and eosin and trichrome-elastin stains were produced on all sections. Tosupport interpretations based on these staining methods,picrosirius red (collagen) and von Kossa staining in addition to immunohistochemistry for smooth muscle cells (Anti-Selected sections were subjected to the following tests: endothelium (Biotinylated Griffonia Simplicifolia Lectin I, Vector Laboratories B-1105, Vector Laboratories, Inc., Burlingame, CA, USA), macrophages (Anti-Lysozyme 12, DAKO A0099[22]), and Smooth Muscle Actin (DAKO M0851).

Digital photomicrographs were acquired, and Image J was used to measure areas and thicknesses. Digital photographs of every part outside the stenosed segments were used to measure the intimal regions. The intimal lesion was assessed in the portion with the biggest intimal area. The most advanced intimal lesion in the stenosis was also graded.

Statistical analysis

Categorical values are presented as counts (frequencies) and numerical values as means (standard deviations). Comparisons of numerical values were performed withthe Kruskal-Wallis test (Stata/IC 10.1 for Windows, StataCorp LP, College Station, TX, USA). A *P*-value <0.05 was considered statistically significant.

RESULTS

One minipig died during follow-up in relation to bloodsampling.

Feeding, body weights, and plasma analyses

All but one minipig (84 kg) weighed between 30 kg and 50 kgat the end of the study. Mean plasma total cholesterol was 6.0 mmol/L (0.3 mmol/L) on standard diet at baseline andpeaked at 21.6 mmol/L (1.3 mmol/L) on the atherogenicdiet, and the rise in total cholesterol was mainly caused bya rise in the low-density lipoprotein fraction [Figure 2].

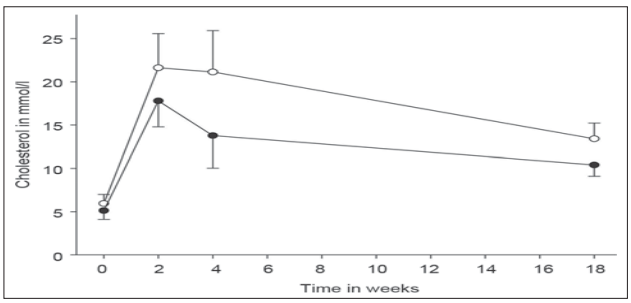

Figure 2: Time course of cholesterol levels. Total (open circles) and low-density lipoprotein (filled circles) cholesterol levels over the courseof the study. Error bars represent standard deviations

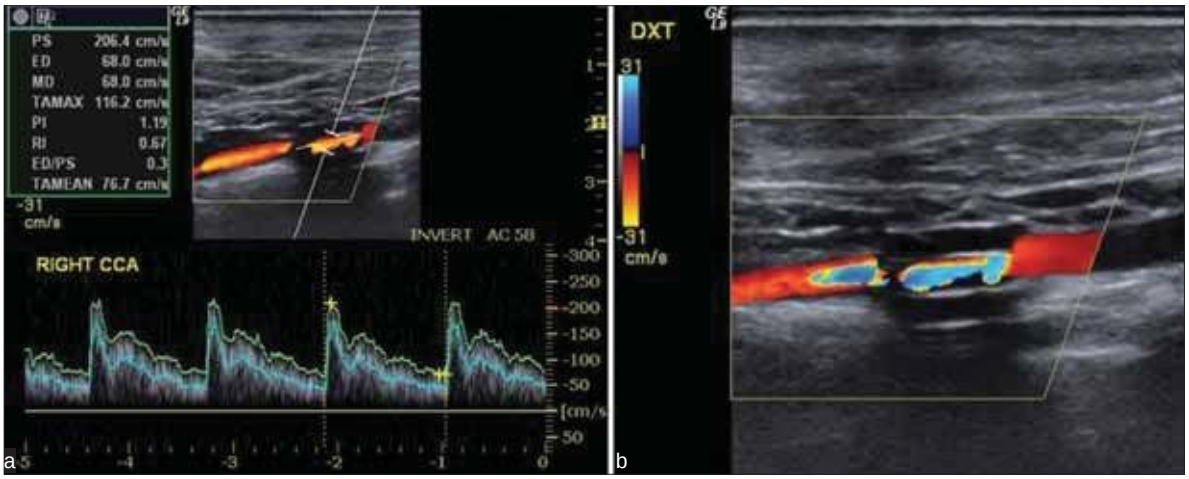

Figure 3: Ultrasound stenosis evaluation in a surgically stenosed carotid artery of a hypercholesterolemic minipig. (a) Peak systolic velocity (PS)206 cm/s in the stenosed segment. (b) Color Doppler showing the proximal segment (right), the stenosed segment, and the distal segment withturbulence (left). The collar material has low echogenicity and the collar is clearly visible around the stenosed segment

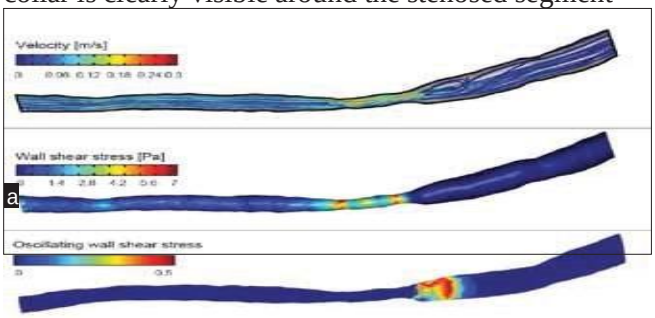

Figure 4: Magnetic resonance imaging stenosis evaluation in a surgically stenosed carotid artery of a hypercholesterolemic minipig. (a) Flow velocity (b) Wall shear stress. (c) Oscillatory wall shear stress; the scale for oscillatory shear stress is from shear stress in one direction only (0) to random shear direction (0.5). This minipig had distal segmentoscillatory wall shear stress with corresponding lesion development

Carotid flow and angiography

The data are presented in Table 1.

One carotid artery was occluded and not included in thecalculated means. Collar placement reduced perioperativetransit time carotid blood flow by 66% (15%). By ultrasound[Figure 3], the stenoses degree was 75% (17%) and the peaksystolic flow velocity in the stenoses was 222 cm/s (91 cm/s).Evaluated with MRI, the stenoses reduced flow by 70% (29%).
One stenosed segment was occluded and not included inthe calculated means. On MRI, the cross-sectional areaswere reduced by 61% (13%) in the stenosed segment compared to the proximal segment. On conventional angiography, the

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stenosis length was 11 mm (1 mm), the diameter stenosis degree was 61% (14%), and the distaldilatation degree was 51% (22%).

Wall shear stress

The data are presented in Table 1 and summarized in Table 2. An example is shown in Figure 4.

Wall shear stress in the three carotid segments was compared to a control segment in the contralateral carotidartery. Among the three carotid segments, wall shear stresswas highest in the stenosed segment and it tended to behigher in the pre-stenotic segment than in the post-stenoticsegment. Oscillatory shear stress was found mainly in the stenotic and post-stenotic segments and was more pronounced in the post-stenotic segments.

Microscopic examination

The data are presented in Table 3.

Thrombosis was the source of the blockage observed in one pig, according to microscopic analysis. Because of the organised thrombus, it was evident that it had happened at the moment the collar was placed. A recanalized thrombus was discovered under a microscope in one of the stenosed segments. It's likely that this thrombus similarly developed just after surgery and then recanalized [Figure 5]. The lumen was patent in the pre- and post-stenotic segments in both of these minipigs. A necrotic core-containing plaque was discovered in both the pre- and post-stenotic sections of the carotid with the blocked stenotic segment.

Table 1: Individual flow and shear stress in nine hypercholesterolemic minipigs with surgically induced carotid stenosis

Lesion	Surgery		Angiography		Ultra-sound	Wall shear stress		Oscillatory shear stress‡			Area with shear stress >0.1‡		
	oscillatory Flow	Stenosis	Pre-Stenosis	Post-stenotic	Stenosis	Post-stenotic	Pre-reduction	Stenosis degree		Post-stenotic degree		Pre-stenotic	
	Stenosis dilatation												
Occluded in stenosed segment*	91	99	0.11	1.18	0.05	0.438	0.396	0.417	100	-	100	100	-
Recanalized thrombus in stenosed segment†	73	85	0.48	6.89	0.3	0.000008	0.0039	0.006	0	0.95	1.96	83	74
Post-stenotic plaque	51	60	-	-	-	-	-	-	-	-	-	42	81
Post-stenotic fatty streak	78	90	0.42	1.24	0.07	0.00002	0.00039	0.0334	0	0.01	11.32	80	18
No atherosclerotic lesion	54	95	1.22	14.85	1.34	0.000012	0.00111	0.00963	0	0.16	2.67	59	38
No atherosclerotic lesion	68	60	-	-	-	-	-	-	-	-	-	47	71
No atherosclerotic lesion	53	65	0.93	8.84	0.65	0.00003	0.00017	0.0122	0	0	3.79	56	38
No atherosclerotic lesion	73	60	0.07	0.21	0.01	0.000014 0.000114		0.000156	0	0	0	58	41

No atherosclerotic lesion	57	60	1.27	3.55	1.14	0.000006 0.000001		0.0000060	0	0	60	48

**An atherosclerotic plaque was found in both the pre-stenotic and the post-stenotic segment. †An atherosclerotic plaque was found in the pre-stenotic and a fatty streak was found in the post-stenotic segment. ‡The scale for oscillatory shear stress is from shear stress in one direction only (0) to random shear direction (0.5)*

Table 2: Summarized wall shear stress and oscillatory wall shear stress in patent carotid arteries

Table 3: Lesion type and thickness in carotid artery segments	Reference Segment	Pre-stenotic	Stenosis	Post-stenotic
Wall shear stress	0.81 (0.42)	0.86 (0.40)	7.07 (5.25)*	0.70 (0.54)
Mean oscillatory wall shear stress ($*10^{-6}$)	50 (50)	15 (10)	1,115 (1,613)	12,246 (12,682)
Relative area with oscillatory wall shear stress >0.1 (%)†	0 (0)	0 (0)	0.22 (0.41)	3.95 (4.35)*

**Different from both proximal segment and reference (P<0.05). †The scale for oscillatory shear stress is from shear stress in one direction only (0) to random shear direction (0.5)*

Segment	Pre-stenotic	Stenosis* (%)	Post-stenotic (%)
Lesion type			
Normal intima or adaptive intimal thickening†	7 7 (100) (78)		5 (56)
Intimal xanthoma (fatty streak)‡	0	0	2 (22)
Plaque without necrotic core§	0	0	1 (11)
Plaque with necrotic core‖	2 (22)	0	1 (11)
Lesion thickness			
Intimal thickness in µm	164 (295)	59 (78)	152 (197)

**n=7: the segments with thrombus were not classified. †Normal connective tissue containing smooth muscle cells. No lipid accumulation or macrophages. ‡Normal intima except foam cell accumulation near lumen. §Extracellular accumulation of lipid and connective tissue with fibrosis with or without calcification. No necrotic core. ‖Contains a necrotic core*

segments. One centimeter into the pre-stenotic segment, the plaque was 432 µm in maximal thickness and occupied 33% of the area within the internal elastic lamina. One centimeter into the post-stenotic segment, the plaque was 463 µm in maximal thickness and occupied 35% of the area within the internal elastic laminae. In the carotid with the recanalized thrombus, there was a plaque with a necrotic core 0.5 cm into the pre-stenotic segment that was 798 µm in thickness and occupied 55% of the area within the internal elastic lamina. In the post-stenotic segment, there was intimal xanthoma (fatty streak) of 351-µm thickness that occupied 6% of the area within the internal elastic lamina.

In the remaining seven minipigs, the stenosed segments showed no signs of thrombus. In these seven minipigs, the pre-stenotic segments had normal intima or adaptive dilatation indicating that low and oscillatory shear stress may have been present in the post-stenotic segment. The other of the two minipigs with atherosclerosis in the post-stenotic segment, had low wall shear stress and a high degree of oscillatory shear stress observed in the patent carotid arteries in its post-stenotic segment. Notably, the post-stenotic wall shear stress was only one- sixth of the wall shear stress in the pre-stenotic segment in this minipig.

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Microscopic examination of a carotid artery stenosis model inhypercholesterolemic minipigs. (a) The stenosed segments with signs of previous thrombosis. (b), (c) and (d). The plaque proximal to the stenosis. Panels a and b are trichrome-elastin stains (collagen blue, smooth muscle cells red, elastin black). (c) Smooth muscle cell actinstain with smooth muscle cells in the fibrous cap covering the necrotic core (asterisk). (d) Macrophage stain with macrophages at the necroticcore border. The scale bar counts for all panels

intimal thickening. In the post-stenotic segments, two hadatherosclerotic lesions. One had a 414-μm thick plaquewithout a necrotic core and one had intimal xanthoma (fattystreak). The remaining five minipigs had normal intima or adaptive intimal thickening in their post-stenotic segments.

Relation between atherosclerotic lesions and **angiography, blood flow and shear stress**

On MRI, the carotid with the occluded stenotic segmenthad very low flow and wall shear stress in the pre-stenotic and post-stenotic segments. However, oscillatory wall shearstress was very high (>0.41 in both segments) and therewere lesions in both segments.

In the carotid with recanalized thrombus, angiography showed a high stenosis degree (83%) and pronounced post-stenotic dilatation (74%). The wall shear stress was lowerin the in post-stenotic segment than in the pre-stenotic segment, and there was more oscillatory shear stress in thepost-stenotic segment. There were atherosclerotic lesions in both segments, but a larger more advanced lesion wasfound in the pre-stenotic segment.

One of the two minipigs with atherosclerosis in the post- stenotic segment did not undergo MRI. Angiographically,this minipig had the largest degree of post-stenotic

DISCUSSION

In common carotid arteries of hypercholesterolemic minipigs, we evaluated the effects of blood flow and wall shear stress on local development of atherosclerotic plaques, including plaques with necrotic cores that are thekey and dangerous characteristic of vulnerable plaques.Atherosclerotic lesions were found in both pre-stenotic andpost-stenotic segments of an occluded carotid and a carotid with recanalized thrombus. In patent carotid arteries, two post-stenotic segments had atherosclerotic lesions, whereasno pre-stenotic segments had atherosclerotic lesions.

The relation between local plaque development and **angiography, blood flow and shear stress**

It has previously been reported that hypercholesterolemic mice and pigs develop atherosclerotic lesions in response to perivascular collar placement.[6-13] In such studies, stenosisevaluation with flow measurements and angiography is not consistently performed. For the first time, we assessed carotid artery flow and geometry with MRI and described local wall shear stress with computational fluid dynamics on this basis. With this detailed description of wall shear stress, we were able to make two interesting observations that at least partially contradict common assumptions.

Firstly, it is often emphasized that the pre-stenotic segment has low endothelial shear stress and this is usedto substantiate that low shear stress promotes plaque development and a vulnerable plaque phenotype.[6] However, we found that endothelial shear stress tended tobe even lower in the post-stenotic segment than in the pre- stenotic segment. This is in agreement with the observed post-stenotic dilatation. With the same volumetric flow inthe pre-stenotic and post-stenotic segments, linear bloodflow velocities and endothelial shear stress should be lowerin a dilated post-stenotic segment. Also, we found that thelow post-stenotic shear stress was often associated with a high degree of oscillatory stress. Hence post-stenotic lesion development seemed as much linked to a high degree ofoscillatory stress as to a low magnitude of wall shear stress.

Secondly, it is often concluded that low shear stress promotes atherosclerotic lesion formation.[5,6] In the present study, we found lesions at locations with the combination of low wall shear stress and oscillatory stress.However, we observed both pre-stenotic and post-stenotic segments without lesions despite the presence low shear stress. These segments were without oscillatory stress. We also observed areas with oscillatory stress and highwall shear stress. These areas, likewise, had not developedlesions. This indicates a combined pro-atherogenic effect of low wall shear stress and a high degree of oscillatorystress that at least exceeds that of low wall shear stress or oscillatory stress alone. The described

dependency on oscillatory stress for atherosclerotic lesion development may explain, at least in part, why plaques are found close tothe stenosis and not distant from the stenosis, even thoughthe same low shear stress prevails here.

Thrombosis may interfere with interpretation

The interpretation of flow, shear, imaging, and microscopic inspection is more complex in stenoses including thrombosis or recanalized thrombosis. A thrombus that originates in the stenosed segment has the potential to spread into the pre- and post-stenotic segments. After several months, it could be challenging to distinguish between a reorganised and endothelialized thrombus and a plaque because they can have similar constituents, such as fibrous tissue, smooth muscle cells, extracellular lipid, red blood cells or their remains, and calcifications.[23, 24] Tissue strands emerging from the plaque into the lumen and perhaps forming a septum that crosses the lumen and connects with the opposing wall should increase the possibility of reorganised thrombus.Moreover, if a thrombus blocks the stenosed section butcessation induced by carotid ligation leads to lesion formation beneath an intact endothelium and without thrombosis. The underlying mechanisms are being studied.[25-28] In humans, the contribution of thrombus to the volume of existing plaques and its organization iswell-described.[23,24]

Limitations

This study was of a relatively short duration, and a highernumber of lesions and more advanced lesions may havedeveloped over a longer study period. Although flow changes were measured perioperatively, it would be preferable to study the minipigs also with ultrasound and MRI earlier to further elucidate the effects of flowon lesion development in this model. The number of minipigs included in this study was limited, so althoughthe study contains some interesting findings that can generate ideas and hypothesis regarding wall shear stress and atherosclerotic plaque development, it did not havethe power to test or prove hypotheses.

A number of assumptions used in computational fluid dynamics introduce limitations to the interpretation of the link between shear stress and lesion development. E.g.,we used the common assumption that arterial compliance could be neglected. However, arterial deformation by the pulse wave, especially as it suddenly meets a severe stenosis,may have effects on atherogenesis. These could also bepart of the explanation for the observation that lesions preferentially form close to a surgically induced stenosis.

CONCLUSION

In hypercholesterolemic minipigs, we induced common carotid blood flow changes with a perivascular collar anddescribe effects of common carotid blood flow and wall shear stress on the development of atherosclerotic lesions.Atherosclerotic lesions were found in both pre-stenoticand post-stenotic segments of a thrombosed carotid anda carotid with recanalized thrombosis. In patent carotidarteries, atherosclerotic lesions were found in the post-stenotic segments only, and development of atherscleroticlesion development seemed dependent on the simultaneouspresence of both low and oscillatory shear stress.

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