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Original Article

Anatomy Section

Early Onset of Atherosclerosis of The Carotid Bifurcation in Newborn Cadavers

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ABSTRACT

Introduction: The anatomy of arterial bifurcations affects blood flow and has a significant role in the development of vascular disease. Therefore, it is important to know the structural characteristics of the Common Carotid Artery (CCA) and its branches for early onset of atherosclerosis in newborns.

Aim: The present study was conducted to evaluate the characteristics of CCA in newborn cadavers.

Materials and Methods: Eight carotid arteries obtained from newborn cadavers were used. The outflow to inflow area ratios was calculated to evaluate vessel diameters. Additionally, scanning electron and light microscopic investigations were conducted with tissue samples. The brachial artery of each cadaver was used as controls. Correlation between area ratios and atherosclerotic endothelial damage was determined.

Results: Light microscopic investigations demonstrated that control group sections showed no positivity for Oil red O staining, while carotid bifurcation regions depicted widespread occurrence of intimal lipid accumulations. Scanning electron microscopic examination of control group sections presented regular endothelial topography, while carotid bifurcation region topography exhibited numerous blood cells and separated endothelial cells. Fibrin accumulation on endothelial surface in low area ratios was another important finding in the examination of its endothelial surface degeneration.

The above-mentioned morphological findings seemed to be quite parallel to outflow to inflow area ratio data favouring low area and degeneration.

Conclusion: The correlation between area ratios and the histological characteristic of cerebral vessels of newborn cadavers indicate that early stages of atherosclerosis began in early embryologic life.

Keywords: Carotid artery, Cerebral blood flow, Cerebral haemodynamics, Endothelium

INTRODUCTION

Atherosclerosis is characterized by a prominent thickening and hardening of blood vessel walls [1-4]. Acquired risk factors such as smoking, high blood pressure, dyslipidemia and diabetes mellitus play role in the development of atherosclerosis [4-10]. Besides these, the genetic factors and the anatomical, histological, and haemodynamic features of the arteries are other factors that predispose the development of atherosclerosis [3-5,11,12].

Blood vessels and their luminal geometry have long been suspected as additional risk factors for atherosclerosis because of their influence on blood flow [9,13-15]. Some arteries are more predisposed for the development of the atherosclerotic plaques [2,4,10,15,16]. Recent studies indicated that the variations in vessel diameter have an impact on the onset of atherosclerosis [5,6,13].

Carotid artery is one of the two vessels that supply blood to brain. Occlusion in the carotid artery leads to degeneration and causes brain symptoms. The anatomical details of the common carotid artery (CCA) and its branches are subject of interest to clinicians and researchers due to their clinical importance and their involvement in plaque formation [7,8,10,13,17-21]. Therefore, it is important to know the anatomical features of the CCA and its branches. CCA divides into two branches i.e. external carotid and internal carotid artery. Atherosclerosis develops primarily at bends and major branches of the arterial network, such as the carotid bifurcation and its branches [2,15,17]. Like other bifurcations of large vessels, carotid bifurcation at the neck region, is more prone to the early development of atherosclerotic plaques [6,14-16]. Flow models suggest that vessel anatomy, in particular vessel diameter and area ratios, affects plaque formation at arterial bifurcations. The carotid bifurcation is one of the most common sites of atherosclerotic plaque [2,9,20,22]. Therefore, assessing the diameters of the CCA, internal and external carotid arteries (ICA and ECA) is important for evaluating the pathological changes [6,22-25].

Despite the progression of atherosclerosis with ageing is widely studied, there are limited studies in newborns. Many studies evaluated the diameters of peripheral vessels in adults, but only few studies were conducted on CCA and its branches in newborns [6,12,19,22]. Hence, an early onset of atherosclerosis is suggested to initiate in the early terms of life. Therefore, our purpose was to investigate the anatomical and histological characteristics of cerebral vessels in newborn cadavers. Thus, we hypothesized that variations in carotid bifurcation lumen geometry would be the self-regulating predictors of ICA atherosclerosis; and we aimed to reveal the early initiation of atherosclerosis in newborn cadavers. The relation between the endothelial damage and outflow to inflow area ratio was also been examined in this study.

MATERIALS AND METHODS

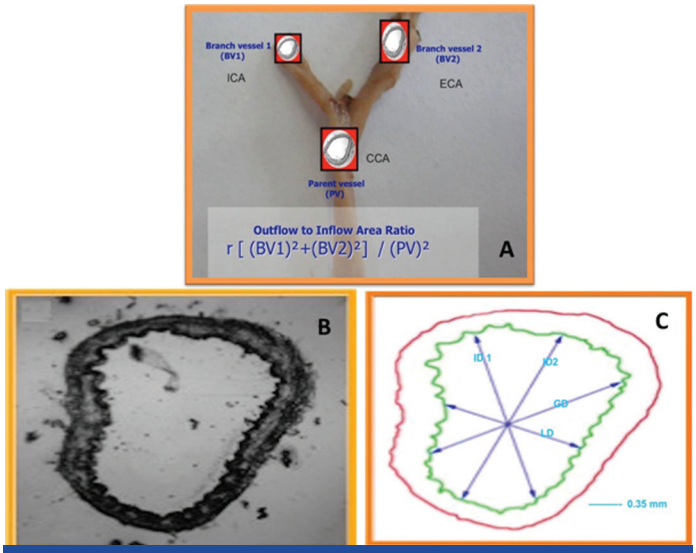
Samples

Eight artery samples (4 right and 4 left) were taken from newborn cadavers which were donated and delivered immediately after death to Marmara University, Department of Anatomy between 2013-2014. All study subjects were about 40 weeks of gestation.

All the studies were carried out in accordance to the ethical guidelines. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

All cadavers were fixed immediately with 5% formalin solution, which was directly injected into cranial, thoracic and abdominal

cavities. Intra-arterial formalin injection has not been done to prevent endothelial damage. Tissue samples taken from branch of CCA were used for both scanning electron microscopy investigations, including calculations for vessel diameters. Samples taken from the ulnar and radial branching sections of the brachial artery of each cadaver was used as controls (these 8 control atherosclerosis-resistant arteries have a similar area ratio with CCA and its branches [Table/Fig-1a-c].



[Table/Fig-1a-c]: a) Schematic presentation of the dissection of the carotid artery. CCA: common carotid artery, ICA: internal carotid artery, ECA: external carotid artery; exposed outflow to inflow area ratio.
b) Sections were photographed using a digital camera under the microscope.
c) The diameters of the arteries were measured. Abbreviations: GD, greatest diameter; LD, least diameter; ID, intermediate diameter, further details in text.

Light microscopy

Cryostat sections of 5 µm were stained with Oil Red O (Sigma-Aldrich, St Louis, Missouri, USA) and counterstained with hematoxylin to indicate depositions of lipid. All groups were examined and evaluated under a light microscope BX 51 (5060 Wide Zoom Olympus®, Tokyo, Japan).

Scanning electron microscopy

For scanning electron microscopy investigations, tissue samples of 1 cm^2 have been fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Merck®, Darmstadt, Germany) for 2 hours and then postfixed in 1% phosphate buffered osmium tetroxide solution (Merck®, Darmstadt, Germany). Samples, dehydrated with increasing series of ethyl alcohol and amyl acetate, have been dried with a Bio-Rad E 3000 critical point dryer. Then re-dried with CPD 010 critical point dryer (CPD 010, Balzers Union, Liechtenstein). Following gold coating with a Sputter Coater (JEOL, Jee-1, Tokyo, Japan), samples were examined under a scanning electron microscope (JEOL 5200 JSM, Tokyo, Japan).

Diameter Calculation Method

Thick transverse sections were cut on a cryostat-microtome at 40 µm from each artery (SLEE, Mainz, Germany). The plane of the cut was perpendicular to the vessel in each case. An eyepiece mm scale (AX0057 24 mm Cross, Olympus, Tokyo, Japan) was used to measure the internal diameters of each artery with an x4 objective and an x10 eyepiece. Three measurements (greatest, least and intermediate diameters) were taken by the same investigator for all measurements, and the average measurement was calculated for each artery. In addition, the outflow to inflow area ratios were calculated according to the formula {(ICA) 2 + (ECA) 2}/(CCA) 2 as mentioned elsewhere [12]. In order to evaluate diameters of vessels, 40 µm cryostat sections were taken and then photographed using a digital camera under the microscope (Spot RT, Sterling Heights, MI, USA). The diameters of the arteries were measured using Image Pro-Plus 6.0 (HP Pavilion dv 4266EA, Palo Alto, CA, USA) [Table/Fig-1].

STATISTICAL ANALYSIS

The data are expressed as mean, standard deviation, median, range, minimum and maximum values. Shapiro Wilk's was used to control whether the distribution of parameters was normal or not. Then correlation between Endothelial Deg. (ED) and Area Ratio (AR) of samples analysed with Spearman Correlation with bootstrap methods. Our sample size is small but we have generalized with bootstrap method. In addition, statistical significance was considered as 5% for all statistical computations. All statistical analysis was performed by R.2.13.0 (R-Project, www.r-project.org, R foundation, Vienna, Austria).

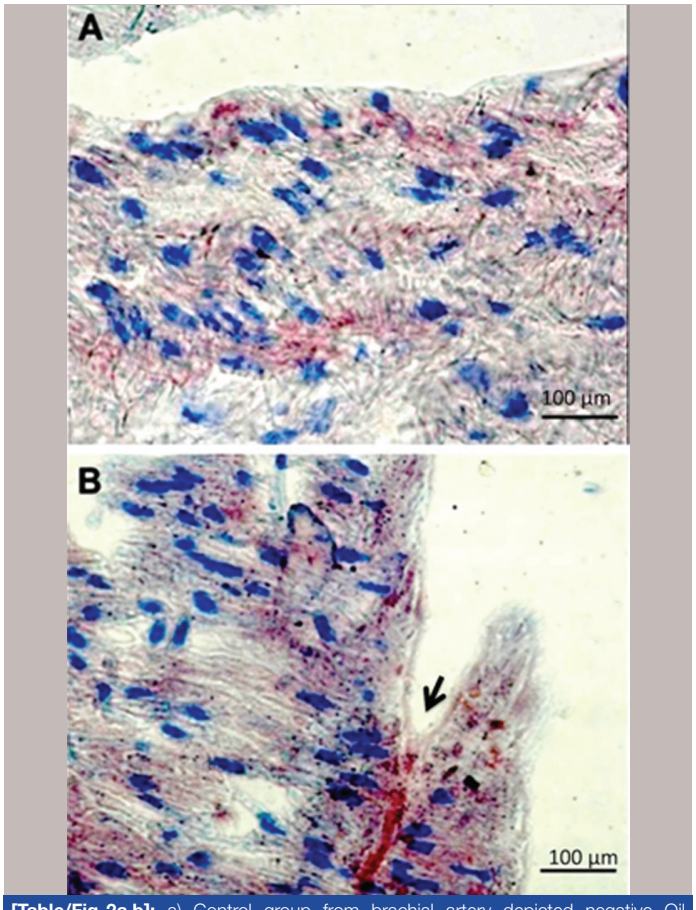
RESULTS

Light microscopy

In light microscopical investigations, control group sections revealed no positivity for Oil red O staining and intimal lipid accumulations [Table/Fig-2a], while morphometric analysis of Oil red O–stained sections of carotid bifurcation regions depicted widespread occurrence of intimal lipid accumulations [Table/Fig-2b].

Scanning electron microscopy

Scanning electron microscopical examination of control group section presented a regular endothelial topography with thin, homogeneously intact, organized endothelial cells [Table/Fig-3a], Slightly disrupted surface topography from the left CCA of the first case [Table/Fig-3b]; swollen endothelial cells on the second case [Table/Fig-3c]; adhesion of numerous blood cells on the endothelium surface, endothelial cells were slightly separated



[Table/Fig-2a,b]: a) Control group from brachial artery depicted negative Oil red O staining without lipid accumulations. Light micrograph: X 400; b) Carotid bifurcation region shows positive Oil red O staining depicted widespread intimal lipid accumulations in this picture. (↓) Light micrograph: X 400, scale bar: 100 µm.

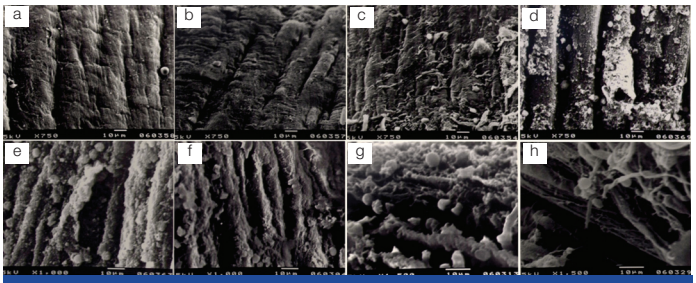
from each other in the third case [Table/Fig-3d]; also endothelial topography showed numerous blood cells stuck on the undulated surface of endothelial cells, which were highly separated from each other on the fourth case [Table/Fig-3e]; highly separated and swollen endothelial cells from the sixth case [Table/Fig-3f]; separated endothelial cells, large pits were full of adhered blood cells from the eighth case [Table/Fig-3g]; On the carotid bifurcation region, fibrin accumulation on the endothelial surface was another important finding in this group describing endothelial surface degeneration from the fifth case [Table/Fig-3h].

Avarage ratio of area for carotid vessels obtained from 8 samples were 0.970 ± 0.14 (range 0.774-1.184) as mean ± SD.

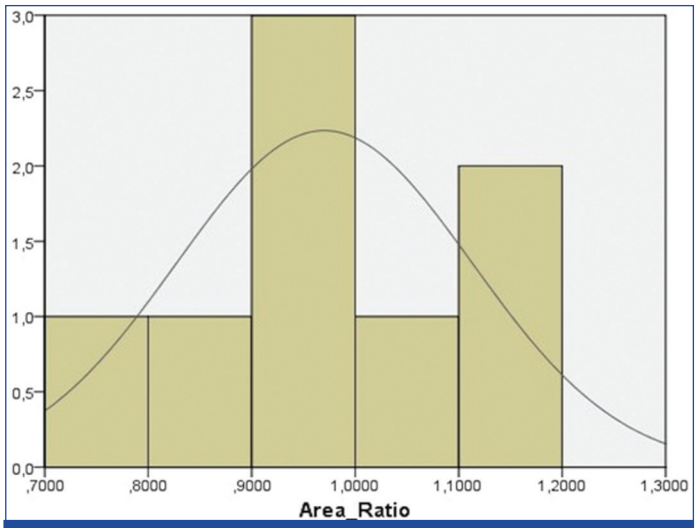
According to our descriptive statistics in [Table/Fig-4], standard deviation of AR was acceptable [Table/Fig-4] indicating that our samples are homogenous for investigating the correlation. Distribution is close to normal distribution. But according to Shapiro Wilk's test results, distribution was not normal. (p=0.024)

Histological scoring systems were set to describe the damage of endothelial cells of vessels. The highest case score was the lower area ratio on the left side of third cadaver (the fifth case) [Table/Fig-5].

Correlation between ED and AR is -75.6% and this correlation were statistically significant. (p=0.30<0.05) [Table/Fig-5] we have used bootstrap methods for generalizing the results. Therefore the confidence interval of this correlation coefficient is



[Table/Fig-3a-h]: a) Control group (brachial artery): Depicted undulated surface, thin endothelial cells, homogeneously intact and organized endothelium; b) Slightly disrupted surface topography of left CCA from the first case; c) Swollen endothelial cells of right CCA from the second case; d) Adhesion of numerous blood cells on the endothelium surface. Endothelial cells are slightly separated from each other. From the left CCA of the third case; e) Depicted highly separated endothelial cells with pit formation and fibrin accumulation. From the right CCA of the fourth case; f) Highly separated and swollen endothelial cells. From the right CCA of the sixth case; g) Endothelial topography is extremely disrupted with typical fibrin formation and separated endothelial cells, large pits full of adhered blood cells, from the right CCA of the eighth case; h) Extremely degenerated endothelium resulting in denuded basal lamina, from the left CCA of the fifth case, scale bar: 10 µm.



[Table/Fig-4]: Histogram of distribution of arterial area ratios indicating that samples are homogenous for investigating the correlation N=8, Xaxis-area ratio Y axis=frequency.

Correlation					Area_Ratio	Endothelial_ Degeneration	Blood_ Adherence	Denuded_Basal_ Lamina
Spearman's rho	Area_Ratio	Correlation Coefficient			1.000	-.756*	-.430	-.548
		Sig. (2-tailed)				.030	.287	.160
		N			8	8	8	8
		Boostrap[c]	Bias		.000	-.003[d]	.046[e]	.040[f]
			Std. Error		.000	.118[d]	.374[e]	.348[f]
			95% Confidence Interval	Lower	1.000	-.907[d]	-.889[e]	-.950[f]
				Upper	1.000	-.584[d]	.521[e]	.304[f]
	Endothelial_ Degeneration	Correlation Coefficient			-.756	1.000	.552	.621
		Sig. (2-tailed)			.030		.156	100
		N			8	8	8	8
		Boostrap[c]	Bias		.003[d]	.000[d]	.018[g]	.020[h]
			Std. Error		.118[d]	.000[d]	.192[g]	.181[h]
			95% Confidence Interval	Lower	.907[d]	1.000[d]	.218[g]	.218[h]
				Upper	.584[d]	1.000[d]	1.000[g]	.956[h]
	Blood_Adherence	Correlation Coefficient			-.430	.552	1.000	.793*
		Sig. (2-tailed)			.287	.156		.019
		N			8	8	8	8
		Boostrap[c]	Bias		.046[e]	.018[g]	.000[e]	-.22[i]
			Std. Error		.374[e]	.192[g]	.000[e]	.150[i]
			95% Confidence Interval	Lower	.889[e]	.218[g]	1.000[e]	.333[i]
				Upper	.521[e]	1.000[g]	1.000[e]	1.000[i]
	Denuded_Basal_ Lamina	Correlation Coefficient			-.548	621	.793*	1.000
		Sig. (2-tailed)			.168	100	.019	
		N			8	8	8	8
		Boostrap[c]	Bias		.040[f]	.020[h]	-.022[f]	.000[f]
			Std. Error		.348[f]	.181[h]	.150[f]	.000[f]
			95% Confidence Interval	Lower	-.950[f]	.218[h]	.333[f]	1.000[f]
				Upper	.304[f]	.956[h]	1.000	1.000[f]

[Table/Fig-5]: Positive Correalation analysis among the variables of carotid vessel samples N=8.
Histological scoring systems were set to describe the damage of endothelial cells of carotid vessels. The highest case score was the lower area ratio on the left side of third cadaver (the fifth case).
*. Correlation is significant at the 0.05 level (2-tailed).
c. Unless otherwise noted, bootstep results are based on 1000 bootstrap samples
d. Based on 884 samples
e. Based on 998 samples
f. Based on 996 samples
g. Based on 882 samples
h. Based on 880 samples
i. Based on 994 samples

-90.7%- -58.4%. Correlation between other the variables are not statistically significant.

DISCUSSION

Atherosclerosis is a series of complex events that can begin in early fetal life [4,16,26]. Its onset has been suggested to be related to numerous risk factors such as genetics, diabetes etc., [3,4,26]. Bifurcation regions such as aorta abdominalis, have been mentioned as primary foci that are prone to atherosclerosis [6,20]. In some studies, it has been suggested that the vessel diameter and area ratios are potentially important determinants of plaque development [9,20,23]. With this information in mind, we extended our previously-published findings [6,17] by examining the characteristics of vessel located in the bifurcation regions [6,11,13,27] in young cadavers.

As indicated in the literature, a vessel diameter and area ratio (score) is around 1.15 [12]. Low ratios could be reflective of increased local stress and endothelial damage. As an expected result, the endothelial response to the damage could be increased permeability along with monocyte adhesion and migration.

The diameters of the CCA, ICA, and ECA have again been measured in the present study. The relative vessel size was shown to be important in the development of the disease [6,11,13,19,27]. Therefore, ECA/CCA, ICA/CCA, ICA/ECA ratios, as well as outflow to inflow area ratio have been calculated. Additionally we evaluated vessels histologically using SEM and histostain to determine the extent to which atherosclerotic pathology was present, if any.

The other main goals of this study were to calculate the mean diameters of CCA, ECA, and relationships between atherosclerosis and ICA and outflow to inflow area ratio in the newborn age group. These data can be of use in intravascular instrumentation and also for understanding the changes in these vessels that occur in fetal life.

The significant intra-individual and inter-individual differences of the carotid artery have already been shown in previous studies [6,9,11,13,16,20,27]. In our current study, we tried to find the answer whether these differences were present early in life.

Atherosclerotic plaque formation has been suggested to be closely related to a decrease in the outflow to inflow area ratio [6]. This fact has been supported in many studies [9,18,21,28-30].

Fisher and Fieman; and Schultz et al., revealed that the bifurcation anatomy influences the blood flow that causes the endothelial damage [9,20]. Mortensen also mentioned endothelial damage and explained that a proportion of a pulse wave arriving at a bifurcation is reflected and the higher the degree of reflection, the more the haemodynamic stress might develop locally. The increase in the pressure could lead to endothelial damage and favour atherosclerotic plaque development [19]. In terms of endothelial damage, our findings showed similar results to the literature.

Early investigations of this geometric risk hypothesis were evaluated in part, owing to relatively small sample sizes. Fisher and Fieman studied the effects of bifurcation angle and area ratio asymmetry on the development of atherosclerosis [6,9,21]. Also, we have limited samples because of the difficulties in obtaining cadavers.

It has been shown that in initial lesions of the atherosclerosis, fatty streaks develop very early in fetal life [4]. The formation of fatty streaks also depends on many factors such as the maternal hypercholesterolemia, the susceptibility of the arteries and genetic factors. The sites of lesion show variability and the fatty streaks tend to occur focally in certain predisposed regions while sparing adjacent unaffected segments [3]. The common carotid and abdominal aorta are much more prone to the development of fatty streaks [4]. The intracranial arteries are less prone to lesion development than extracranial arteries; therefore the early lesions develop in extracranial arteries rather than intracranial arteries [4].

The reason why some of the arteries are more susceptible to atherosclerotic changes is not well understood/ established, but the haemodynamic factors and morphologic features of the artery may play a role [9,12,14,20,21]. We focused on the carotid bifurcation.

Shultz et al., findings [16] show that variation in carotid bifurcation anatomy is not restricted to differences in absolute vessel size. In addition, vessel diameter and area ratios vary markedly between and within individuals [20]. Our findings also partly confirmed these studies.

Previous studies which have studied the relation between bifurcation geometry and the presence of cerebral artery aneurysms on angiographic images have localized atherosclerotic lesions at the bifurcations of human cerebral arteries on autopsy cases, but in this study, there were no available data on the endothelial topography in bifurcation geometry of new born cadavers in the common carotid artery and its major branches. For this reason histologic evaluation makes our study more valuable [31].

Gosling et al., calculated the optimal area ratio of an arterial bifurcation, causing the least reflection of pressure to be 1.15. That ratio can be close to ideal in human infants however, in the long term, the decrease in outflow to inflow area ratio can lead to atherosclerotic plaque formation. Gosling et al., studied 19 cases, with ages ranging from 0-10 and the outflow to inflow area ratio was found to be 1.11±0.02 at 0 age group. Our results were closer to the optimum ratio [12].

Sitzer et al., did attempt to provide a mechanistic link by suggesting that their angle or rotation of ICA origin may be related to the ICA angle of insertion (comparable with the ICA–CCA angle of Lee et al.,), which has been linked to flow disturbances. In our study, we did not evaluate any rotation or angle [2,25].

There are numerous studies on the diameters of CCA, ICA and ECA in adults, but few studies on newborns. To our knowledge there are no earlier data available on the relationship between of diameter of newborn cadavers and the CCA, ICA and ECA. Subsequently, we correlated our data with our earlier studies that measured the internal diameters [6].

Sehirli et al., reported the mean outflow to inflow area ratio as 1.18 ± 0.22 mm in male and 1.10 ± 0.33 mm in female newborn cadavers for the common carotid artery bifurcation [6]. The results of the present study on intracranial bifurcations show that the means of outflow to inflow area ratio in fetal material are close to the optimum value in fetal material for the cerebral vessels.

Consistent with our results, the lumen geometry of arterial bifurcations influences the blood flow that causes endothelial damage [9,13,26,32].

Potential Limitations and Implications

Our geometric variables were strong, but not perfect as our study was relatively small, retrospective, and focused on a limited number of newborn cadavers. Specimen availability was limited for both affected groups and controls. It should also be noted that our study was not intended to be an epidemiological study, and therefore our groups did not represent the characteristics of a wide-ranging population. Our detection of blood cells and fibrin on the surface of the endothelium are interpreted as pathological findings. However, there might be problems with the poor fixation of specimens i.e. blood could not be washed out from the arterial lumen before specimen fixation. Finally, Scanning Electron microscopy (SEM) studies can be associated with various kinds of artifacts. We therefore hope to confirm our pathological findings using transmission electron microscopy in future studies. Despite the above potential limitations, the current study seems

to set a modest upper bound on the influence of local versus known or unknown systemic cardiovascular risk factors on wall setting. Thus, our findings are consistent with the previous ones to support theory that carotid bifurcation geometry and/ or local haemodynamics) is a risk factor for early carotid wall thickening.

CONCLUSION

The results showed that the outflow to inflow area ratio was very close to optimum in newborns. These data can be helpful for understanding the anatomical changes of the CCA, ICA, and ECA; and the correlations between area ratios and the histologic evaluations of cerebral vessels of newborn cadavers indicate that the early stage of atherosclerosis began in early embryologic life. Our findings support the hypothesis that carotid bifurcation anatomy is a risk factor for the early onset of atheroma plaques. However, further studies are needed to highlight the other factors and mechanisms.

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