Age of Collagen in Intracranial Saccular Aneurysms

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Background and Purpose—The chronological development and natural history of cerebral aneurysms (CAs) remain incompletely understood. We used $^{14}$C birth dating of a main constituent of CAs, that is, collagen type I, as an indicator for biosynthesis and turnover of collagen in CAs in relation to human cerebral arteries to investigate this further.

Methods—Forty-six ruptured and unruptured CA samples from 43 patients and 10 cadaveric human cerebral arteries were obtained. The age of collagen, extracted and purified from excised CAs, was estimated using $^{14}$C birth dating and correlated with CA and patient characteristics, including the history of risk factors associated with atherosclerosis and potentially aneurysm growth and rupture.

Results—Nearly all CA samples contained collagen type I, which was $<$5 years old, irrespective of patient age, aneurysm size, morphology, or rupture status. However, CAs from patients with a history of risk factors (smoking or hypertension) contained significantly younger collagen than CAs from patients with no risk factors (mean, 1.6±1.2 versus 3.9±3.3 years, respectively; P=0.012). CAs and cerebral arteries did not share a dominant structural protein, such as collagen type I, which would allow comparison of their collagen turnover.

Conclusions—The abundant amount of relatively young collagen type I in CAs suggests that there is an ongoing collagen remodeling in aneurysms, which is significantly more rapid in patients with risk factors. These findings challenge the concept that CAs are present for decades and that they undergo only sporadic episodes of structural change. (Stroke. 2014;45:1757-1763.)

Key Words: intracranial aneurysms • natural history • radiocarbon dating • risk factors

The prevalence of unruptured cerebral saccular aneurysms (CAs) in the general population is 2% to 3%.1 CAs can remain clinically silent, present with symptoms of mass effect, or they can rupture, causing subarachnoid hemorrhage or hemorrhage into other brain compartments. The high case fatality rate of subarachnoid hemorrhage of $\leq 35\%$ has stimulated interest in understanding the formation and natural history of these lesions, to define standards for screening or prophylactic treatment and to identify those CAs that are at increased risk of rupture.2,3 One hypothesis suggests that CAs form and then grow at a constant rate.4 Alternatively, CA growth has been noted to alternate stochastically between periods of stability and instability or growth, during which they are prone to rupture.5 Nevertheless, no compelling biological evidence exists to support either theory because most data are derived from mathematical modeling studies. One possibility to investigate the chronological development of CAs better is radiocarbon birth dating of collagen type I, which is the most dominant molecular constituent of CAs.

Radiocarbon birth dating takes advantage of the sharp increase and subsequent slow attenuation of atmospheric $^{14}$CO$_2$ concentrations that occurred after above ground nuclear bomb tests between 1955 and 1963. After the implementation of the Limited Test Ban Treaty, there was no additional significant elevation of atmospheric $^{14}$CO$_2$, resulting in a nearly exponential decrease of atmospheric $^{14}$CO$_2$ levels, not because of $^{14}$C radioactive decay (half-life, 5730 years) but instead because of $^{14}$CO$_2$ diffusion from the atmosphere and equilibration with the oceans and biosphere.6,7 Therefore, the consumption of plants and of animals that live in plants results in $^{14}$C levels in the human body parallel to those in the atmosphere.8 $^{14}$C is integrated into any human biomolecule or genomic product in proportion to the atmospheric $^{14}$C level at the time of formation. Thus, a measurement of $^{14}$C in the collagen type I of CAs allows estimation of the age of the collagen.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.114.005461/-/DC1.
DNA, reflecting the F\textsuperscript{14}C level in the atmosphere at the time the biomolecule was synthesized.\textsuperscript{8} However, F\textsuperscript{14}C content can be measured in DNA to birth date human cells, and birth dating of proteins can be used to estimate development and turnover of pathological structures.\textsuperscript{9,10} We previously reported on the feasibility of this method for CAs, provided that the extracted CA collagen was pure and not mixed with other proteins.\textsuperscript{11} However, the small sample size and the lack of human artery control tissues did not permit more definitive conclusions based on this preliminary data. Here, we investigated the age of CA collagen as an indicator for collagen turnover in a distinctly larger patient cohort to identify determinants for aneurysm development and the potential relationship with collagen turnover in human cerebral arteries.

### Materials and Methods

For details on CA and cerebral artery sample processing, as well as birth dating of CA collagen, see the online-only Data Supplement.

### Ethics Committee Approval

The research ethics committees of the Medical Faculty of Heinrich-Heine University, Düsseldorf, Germany (ID 3365), St. Michael’s Hospital, Toronto, Canada (ID 09-309), and Lawrence Livermore National Laboratory, Livermore, CA (ID 10–108) approved this study.

### Sample and Data Collection

Tissue from ruptured and unruptured CAs was collected from patients undergoing surgical repair of their CA. Patients were included if (1) the CA diameter was ≥5 mm, (2) they were scheduled to undergo neurosurgical clipping, (2) the operating neurosurgeon considered it medically indicated or safe to resect a portion of the CA after clipping, and (4) informed consent was obtained for scientific data analysis. Patients were excluded (1) if they were treated for a CA <5 mm and (2) when CA sampling was not safe or technically feasible. For further analysis, data on potential risk factors for CA formation/rupture (ie, hypertension, cigarette smoking, or cocaine consumption) were recorded. CA location, irregularity, diameter, and other size measurements were assessed by neuroradiologists blinded to further analyses. After surgical removal, CA domes were placed in sterile containers and frozen at –80°C until subjected to collagen purification. In 3 larger CAs, 2 separate samples were retrieved to confirm \textsuperscript{14}C intercepts within different parts of these CAs for internal validation. Ten samples from human cerebral and 2 samples from extracerebral arteries from 5 cadavers and 5 collagen samples of known age from newborn mouse tendons served as controls. For the cadaveric cerebral arteries, 5 samples were obtained from the proximal and intradural portion of the internal carotid artery and 5 samples from the distal middle cerebral artery.

### Definitions

CA size was defined as the greatest CA diameter, measured using 3-dimensional (3D) reconstruction of the catheter angiograms. CA irregularity was defined as multiple lobes, presence of daughter-sac, or significant irregularity of the CA wall on the 3D angiographic reconstruction images. Aspect ratio was defined as the ratio of CA neck width and CA largest diameter.\textsuperscript{12} The presence of risk factors potentially associated with an increased risk of CA rupture was assessed during the first clinical presentation or after admission because of subarachnoid hemorrhage.\textsuperscript{2,13} Hypertension, irrespective of whether it was treated or untreated, was defined as systolic blood pressures >140 mm Hg or diastolic blood pressure >90 mm Hg on admission or a previous diagnosis of hypertension. Current cigarette smoking was defined as a risk factor for adults who had smoked ≥100 cigarettes in their lifetime and smoked cigarettes every day (daily) or some days (nondaily) at the time of clinical presentation. Cocaine use was considered as a potential risk factor if used within 1 year of clinical presentation.

### Statistical Analysis

Pearson product-moment correlation was used to correlate continuous variables, such as patient age and morphological CA measurements with CA F\textsuperscript{14}C levels and estimated collagen age. Spearman correlation was used to analyze correlations between dichotomized variables (CA rupture status, irregularity, or presence of risk factors) and continuous variables (estimated collagen age and F\textsuperscript{14}C levels). Differences between groups were compared by the nonparametric Mann-Whitney test to account for small group sizes. Correlations and differences were always analyzed for both F\textsuperscript{14}C levels and estimated CA collagen age to avoid overinterpretation of single results because of sample size. For statistical analysis, risk factors were classified and defined as any risk factor versus no risk factor. The odds ratio for patients to harbor a CA with an estimated collagen age of ≤1 year was calculated for patients with risk factors when compared with those without. Significance was accepted at a level of P≤0.05. All statistical analysis was performed using SPSS version 15.0.1 (Ulead Technologies, Chicago, IL).

### Results

Between March 2009 and April 2013, a total of 293 CAs were surgically repaired at the institution of first author. During this period, 53 CA samples from 50 patients met the inclusion criteria and were collected. None of the CAs was known to have existed for a long time (ie, ≥2 months before aneurysm repair). Ultimately, samples from 36 ruptured and 10 unruptured CAs from 43 patients yielded sufficient amounts of collagen for further accelerator mass spectrometry analysis (Table I in the online-only Data Supplement). Mean patient age in this cohort was 55.1±11.5 years. Gel electrophoresis confirmed the high purity of the collagen from CA samples, with collagen type I and lesser amounts of collagen type V being the only detectable components (Figure 1). Despite some variability in the amount of purified collagen after pepsin digestion, there was no association between the yield of collagen from a CA and its F\textsuperscript{14}C measurements. Importantly, CAs and cerebral or extracerebral arteries did not share a dominant structural protein, which could be used to compare collagen age and turnover in normal arteries and CAs. Gel electrophoresis of the pepsin-digested human cerebral arteries revealed no substantial enrichment of collagen type I or V, but several unidentified proteins, or fragments thereof, which were stable against further digestion with pepsin (Figure 1). Because of their low amounts of collagen type I, the normal cerebral arteries were not amenable to birth dating of collagen type I. The 5 control collagen samples from mouse tendons confirmed the validity of the method because all of these samples gave F\textsuperscript{14}C values for collagen, corresponding to the F\textsuperscript{14}C values of the mouse chow fed to the animals (data not shown).

### Birth Dating of Collagen From Cerebral Aneurysms

There was a distinct dissociation between the patients’ years of birth and age intercepts of their corresponding CAs (Figure 2; Table I in the online-only Data Supplement). Except for 3 samples (samples 8, 30, and 33), all CA collagen samples were ≤5 years old (overall median age estimate, 1.5 years; interquartile range, 2.5 years). There was no difference in the estimated age (P=0.946) or F\textsuperscript{14}C levels (P=0.739) of collagen...
from ruptured and unruptured CAs (Figure 3). However, CAs from patients with a history of potential risk factors for CA formation and rupture (hypertension, cigarette smoking, or cocaine use) contained significantly younger collagen. Mean collagen age was 1.6±1.2 years for patients with risk factors when compared with 3.9±3.3 years for patients without risk factors (P=0.012; Figure 3). This difference was also reflected in the actual F14C levels (1.043±0.0106 for patients with risk factors versus 1.0563±0.0207 for patients without risk factors; P=0.027). Patients with a history of risk factors were more likely (odds ratio, 4.29; 95% confidence interval, 0.98–18.72; P=0.053) to have CA collagen <1 year when compared with patients without risk factors. The modest inverse correlation between patient age and estimated collagen age (P=0.022) was not confirmed after the correlation with actual F14C levels (P=0.115; Figure 3). There was no association between estimated CA collagen age and CA characteristics, with nonsignificant correlations of estimated CA collagen age and largest CA diameter (r=–0.005; P=0.974), CA irregularity (r=–0.08; P=0.598), and aspect ratio (r=–0.005; P=0.971).
Figure 2. Birth dating of collagen in cerebral aneurysms (CAs). A, Atmospheric $^{14}$CO$_2$ levels are illustrated for the past 2000 years. These have been relatively stable except for a large increase between 1955 and 1963 because of atmospheric nuclear testing; the resulting spike of atmospheric $^{14}$CO$_2$ is called the bomb pulse. The $\Delta^{13}$C nomenclature corrects for radioactive decay and shows the historical $^{14}$CO$_2$ production rate. Note the change in temporal resolution after 1945 for illustration purposes. B, The birth dates of patients’ (vertical lines) and CA age intercepts (horizontal lines) are projected onto the $F^{14}$C bomb pulse curve, illustrating the dissociation between the patient age and the estimated CA age. C, CA age intercepts (horizontal lines) in relation to time of sample acquisition for ruptured (●) and unruptured (○) CAs.
Discussion

We used $^{14}$C birth dating of collagen extracted from CAs as a measure of collagen age and turnover to demonstrate that CAs invariably contain recently formed collagen type I or that they are dynamic structures containing collagen that is typically ≤5 years young. The age of collagen in CAs is independent of CA clinical presentation (ruptured versus incidental) and, moreover, of factors that may be associated with CA formation or rupture (ie, patient age, aneurysm size, and morphology). Importantly, we could not compare the age and turnover of collagen type I in CAs with that in cerebral arteries because CAs and normal arteries of the circle of Willis did not share a representative and exclusive structural protein, such as collagen type I.

Previous observational data on a selected cohort of patients with unruptured CAs suggested that CA location in the posterior circulation and increased CA size are significantly associated with risk of rupture and, moreover, that small, unruptured CAs have a low risk of rupture. Higher rates of rupture, but still highly correlated with CA size, were reported in another series from Japan. The discrepancy between the low risk of rupture of small, incidentally detected CAs in previous observational studies and the high proportion of small CAs in patients with subarachnoid hemorrhage has been difficult to reconcile. The prevailing concept remains that there are different populations of small CAs, including those that grow and reach a stable size, those that continue to enlarge and either reach a stable larger size or rupture over the long term, and those that form, enlarge, and then rupture over a relatively short period of time. Aside from our preliminary birth dating study in a small patient cohort, data to support or challenge this concept are mainly derived from animal studies or mathematical models and observational studies of human CAs. In addition, the existing knowledge on collagen turnover in arteries is mainly derived from animal models or extracranial vessels. In general, increased overall collagen turnover in extracranial arteries has been reported under conditions inducing arterial remodeling, but studies have not differentiated turnover of specific collagen types and have rarely examined human cerebral arteries. Thus, biological data on collagen age and turnover in larger human CA cohorts in relation to cerebral arteries are lacking.

In line with our preliminary report, the most likely explanation of our current findings is that there is an ongoing collagen

Figure 3. Determinants for cerebral aneurysm (CA) collagen age. A, Estimated collagen age for patients with ruptured and unruptured CAs. ○, outliers; ●, extreme outliers. B, Estimated CA collagen age for patients with and without a history of risk factors for aneurysm formation and rupture. ○, outliers; ●, extreme outliers. C and D, Correlation of patient age with estimated CA collagen age or actual $^{14}$C levels in CA collagen, respectively. Correlations remained significant after exclusion of the extreme outlier. $^* P=0.012.$
type I biosynthesis in CAs because of dynamic remodeling comparable with a continuous attempt for fibrotic tissue repair to a previous event causing vessel wall instability and the aneurysm formation.11,26–28 Interestingly, this remodeling seems to be significantly accelerated in patients with risk factors associated with atherosclerosis and potentially aneurysm growth and rupture (hypertension, cigarette smoking, or cocaine use), possibly because of increased hemodynamic stress.13,18,29 This is consistent with serial imaging data, showing that some unruptured CAs do undergo growth, including selected smaller aneurysms and a substantial proportion of larger aneurysms especially in patients who smoke.30,31 Some unruptured CAs may grow for short times punctuated by long periods of nongrowth, suggesting that collagen remodeling is ongoing and dynamic.5,30 Thus, variable, nonlinear growth rates of some CAs may also better explain why ruptured CAs are usually small and would not be predicted to rupture if found incidentally.7,15,18 Ultimately, our findings also underline the extracellular matrix and the biomechanical properties of CAs and cerebral arteries are fundamentally different. Although collagen type I is a main constituent of the CA wall, mechanical stability in cerebral arteries is maintained by a complex network of elastin; collagen types III, IV, and VI; and smooth muscle cells but without a significant contribution of collagen I. In addition, cerebral arteries do not comprise a large tunica adventitia as a fibrous tissue component, which explains the relative absence of collagen type I.22,25

There are some limitations to our findings. Because of the cross-sectional nature of our study and the lack of CA samples that have been followed up for many years, we cannot draw definite causal inferences about the actual chronological onset of aneurysm formation or a potential association with a heightened risk of CA rupture. Furthermore, it cannot be ascertained whether the age of collagen is different in the neck or in the remaining CA tissue. In addition, we can only estimate relative collagen turnover using radiocarbon birth dating as an indicator because the total collagen content in human CA samples cannot be monitored over time.33 However, because collagen I is the main constituent of CAs and because we generally excised and analyzed ≥2/3 of the aneurysm dome, the birth dating measurements are likely to reflect the majority of the aneurysmal mass. Furthermore, the measurement precision in radiocarbon birth dating corresponds to a chronological uncertainty of 1 to 3 years, and current technology does not allow for better temporal resolution in samples. Finally, the extractability of collagens from different CAs was variable. However, although the extractable, thus analyzable collagen I fraction varied from 11% to 100% (mean, 41±26%) of the total collagen content in the CA, F14C levels throughout all samples were consistent.

In summary, the abundance of young age of collagen in CAs indicates that structural remodeling of collagen in CAs is an ongoing and dynamic process. This process seems to be more rapid in the presence of risk factors, such as hypertension and cigarette smoking. Because of fundamentally different biochemical composition of CAs and cerebral arteries, these data cannot be directly compared or extrapolated with the normal cerebral vasculature. However, our data suggest that collagen in CAs, irrespective of their size or rupture status, has developed rather recently in the life of a patient. Ultimately and, in line with more recent data on serial aneurysm imaging, our data imply that patients with conservatively managed, incidental CAs and concomitant risk factors should be advised for strict control and effective modification of the risk factors.

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Disclosures

None.

References

Sample Processing and Purification

Collagen purification was performed under sterile conditions in a laboratory with no contamination with $^{14}$C in order to avoid sample contamination and false increase of $^{14}$C levels. For isolation of collagen from CAs, cerebral arteries and mouse tendons, tissue fragments were minced with a scalpel to increase protease accessibility. Tissue fragments were suspended in at least 1 mL of 20 mmol/L HCl. Collagens were extracted after digestion with 100 µg of pepsin/g of tissue at 4°C for 72 hours and were precipitated for 6 hours after addition of solid NaCl to a final concentration of 2.5 mol/L. After centrifugation, collagen was re-dissolved in 20 mmol/L HCl and exhaustively dialyzed against 20 mmol/L HCl. The dialysate was lyophilized using a Concentrator Plus ® (Eppendorf, Hamburg, Germany) and subjected to birth dating by $^{14}$C-accelerator mass spectrometry (AMS). For selected samples, collagen content and purity were assessed by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and residual tissue fragments after pepsin digestion were used to quantify the amount of remaining collagens using the total collagen assay (Quickzyme biosciences, Leiden, Netherlands) according to the manufacturer’s instructions.

Birth Dating of CA Collagen

All $^{14}$C-AMS analyses were performed blinded to patient and CA-related data. Purified collagen samples were transferred to 6 mm O.D. quartz combustion tubes and lyophilized. Excess copper oxide was added to each dry sample and tubes were evacuated and sealed with a H$_2$/O$_2$ torch. Samples were combusted at 900°C for 3.5 hours and allowed to cool to room temperature overnight. The evolved CO$_2$ from each sample was cryogenically purified, trapped and reduced to graphite in the presence of iron catalyst in individual reactors. The $^{14}$C/C concentration in the graphite was measured at the Centre for AMS, Lawrence Livermore National Laboratory using the 10 MV High Voltage Engineering Europa FN-class tandem electrostatic AMS spectrometer with standard measurement protocols. The collagen samples were measured for 30,000 $^{14}$C counts per cycle for five to eight cycle repetitions to achieve measurement errors within 0·3-0·8%. Corrections for background contamination of fossil and contemporary carbon introduced during AMS sample preparation were made using standard procedures. The concentration of $^{14}$C/C was expressed using the F$^{14}$C nomenclature ± 1 standard deviation (SD). The intercept date range corresponds to the two
SD range of atmospheric $^{14}$C/C mapped onto the chronological record, corresponding to a chronological uncertainty of one to three years in most cases. Age of CA collagen was estimated based on intercept date ranges.

**SUPPLEMENTAL REFERENCES**


## SUPPLEMENTAL Table I

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**Epidemiological data, radiological and chronological aneurysm measurements for every patient and CA.** The incidence of potential risk factors for aneurysm formation and rupture, such as hypertension, cigarette smoking, cocaine use or their combination were concluded and dichotomized (yes vs. no). The chronological range of each CA depends upon the measurement precision and slope of the bomb curve at the intercept. Abbreviations: ACA indicates Anterior Cerebral Artery; AR, aspect ratio (aneurysm dome/neck diameter); ACom, Anterior Communicating Artery; F, female; M, male; MCA, Middle Cerebral Artery; PCom, Posterior Communicating Artery; PICA, Posterior Inferior Cerebral Artery; PCA, Posterior Cerebral Artery.