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CLINICAL RESEARCH

Human Atherosclerotic Plaque Progression Is Dependent on Apoptosis According to Bomb-Pulse ^{14}C Dating

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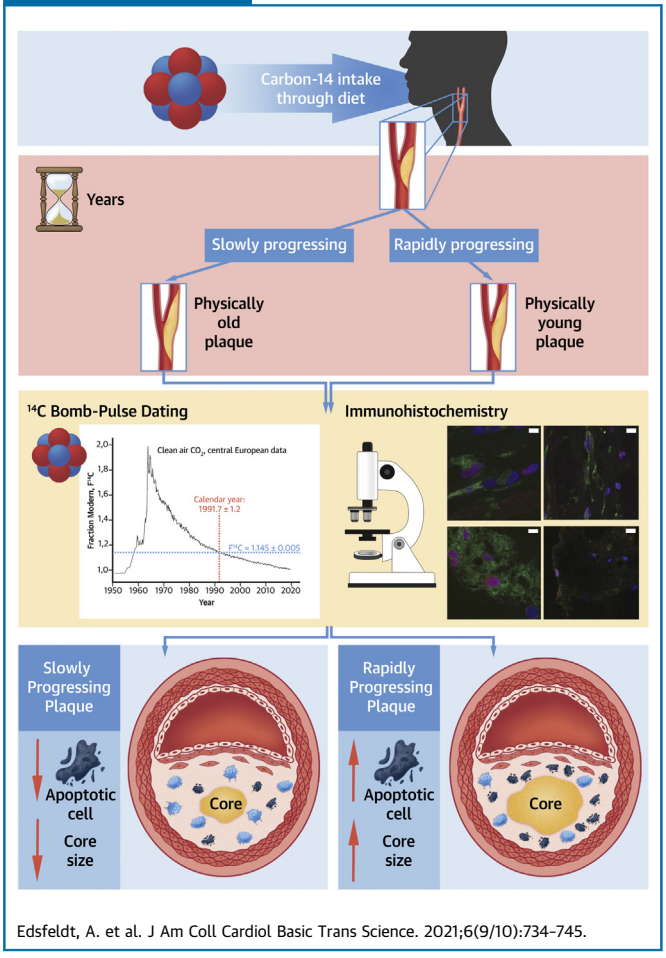
VISUAL ABSTRACT



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HIGHLIGHTS

- **Individuals with rapidly progressing atherosclerotic plaques are at higher risk to experience acute complications. Using a ^{14}C bomb-pulse dating method, we explored the importance of different biological components for the timeframe of plaque progression in human atherosclerosis.**
- **According to the ^{14}C bomb-pulse dating method, increased apoptosis was the main component associated with a young physical plaque age, reflecting a rapid progression.**
- **Physically young atherosclerotic plaques also had more apoptotic cells and larger cores than physically old plaques.**
- **Our findings in combination with recent advances in imaging techniques could guide future diagnostic imaging strategies to identify rapidly progressing plaques or therapeutic targets, halting plaque progression.**

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ABSTRACT

Individuals with rapidly progressing atherosclerotic plaques are at higher risk of experiencing acute complications. Currently, we lack knowledge regarding factors in human plaque that cause rapid progression. Using the ^{14}C bomb-pulse dating method, we assessed the physical age of atherosclerotic plaques and which biological processes were associated with rapidly progressing plaques. Interestingly, increased apoptosis was the main component associated with a young physical plaque age, reflecting rapid plaque progression. Our findings in combination with recent advances in imaging techniques could guide future diagnostic imaging strategies to identify rapidly progressing plaques or therapeutic targets, halting plaque progression.

ABBREVIATIONS AND ACRONYMS

AMS = accelerator mass spectrometry

IQR = interquartile range

PCNA = proliferating-cell nuclear antigen

SMC = smooth muscle cell

TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling

Atherosclerosis is a progressive inflammatory chronic disease, leading to the formation of plaques in the innermost part of the arterial wall (1,2). Autopsy studies suggested that atherosclerotic plaques develop over many years. Similarly, and for the first time in living subjects to our knowledge, we previously demonstrated that most of the plaque tissue is actually formed 10 or more years before diagnosis (3). Large plaques or those with a high degree of stenosis are more likely to cause clinical manifestations, and a rapid plaque progression is a crucial step in this process (4-7). The identification of fast-progressing plaques has been challenging and is a great clinical unmet need, which could provide a window of opportunity to ideally initiate treatment to halt plaque progression in time, in a personalized way. Recent imaging studies have provided evidence that plaques could progress either slowly or rapidly and that specific plaque characteristics could predict fast-progressing plaques (7,8). The identification of such features that could be assessed by imaging technologies would be of great clinical interest to identify high-risk individuals and to intensify their medical treatment in time to halt the progression. However, the actual timeframe of the underlying tissue biology in rapidly and slowly progressing plaques has not been possible to assess.

To study the progression of pathologic tissues, such as plaques, dating methods are essential to determine the biological age of the specimens. This has been done in many fields, such as archaeology and enology, by using the ^{14}C bomb-pulse dating technique. This approach is based on the premise that the ^{14}C concentration in human cells and tissues closely parallels the ^{14}C concentration in the atmosphere at the time of their formation (**Figures 1A and 1B**) (9,10). In this way, it is possible to detect the physical age of the plaque, therefore assessing how fast it has progressed in a living patient.

The plaque is generally composed of inflammatory cells, often macrophages, and a lipid-rich necrotic core covered by a fibrous cap with smooth muscle cells (SMCs) (1). In plaques, several processes are happening simultaneously, such as lipid accumulation, cell proliferation, apoptosis, extracellular matrix degradation, and repair (1,2). The balance among these processes is essential for plaque progression and clinical outcome (1,6,8). Which of these processes underlies fast plaque progression is still unknown.

In this study using the ^{14}C bomb-pulse dating method, we first stratified plaques from living patients according to physical plaque age and then used fluorescent and histochemical analyses to characterize the biological components and the 6 most common processes within the plaques. We showed that of all the studied components, only apoptosis (particularly of macrophages and SMCs) was significantly associated with a younger physical plaque age. These findings support the importance of apoptosis in plaque progression, unravelling its potential use for diagnostic and therapeutic strategies for patients with fast-progressing plaques.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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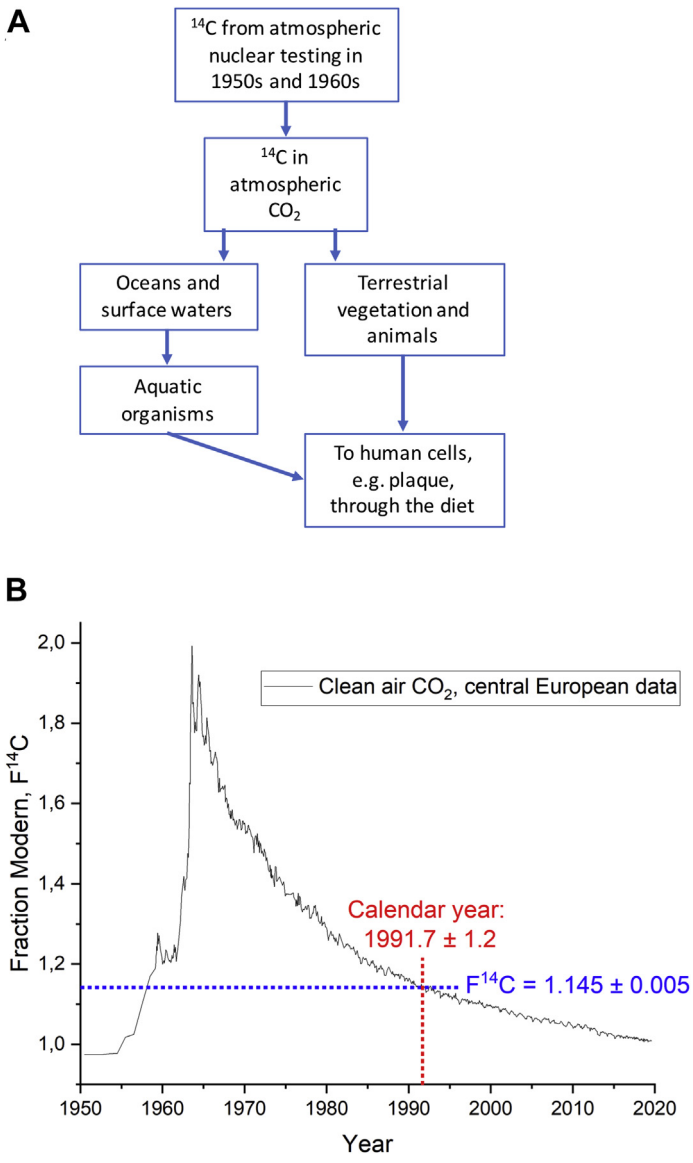
FIGURE 1 Description of ^{14}C Bomb-Pulse Dating



(A) Atmospheric testing of nuclear weapons during the late 1950s and early 1960s generated large amounts of ^{14}C, which was spread throughout the atmosphere as CO_2. Bomb ^{14}C mainly enters the human body through the diet. **(B)** The highest ^{14}C level in atmospheric CO_2 was observed in 1963, when the specific activity of ^{14}C in carbon in the atmosphere was approximately twice that of the natural level (expressed in units of fraction modern [$F^{14}C$], approximately equal to 1.0 before the nuclear weapon testing). Since the testing ban in 1963, atmospheric ^{14}C-specific activity has decreased because of the CO_2 uptake by the oceans and biosphere and because of the fossil fuel ^{14}C-free CO_2 input. The ^{14}C activity concentration in the human diet approximately follows the atmospheric curve with some delay (36). The average age of carbon in the tissue can be estimated by analyzing its ^{14}C content, as visualized in the Figure. The uncertainty of the ^{14}C measurement leads to a corresponding uncertainty in the age calibration. The typical uncertainty is 0.5% to 1% and is mainly based on the number of atoms that have been counted. We define the physical age of a tissue sample as the difference in years between the calendar date of surgery and the calendar date obtained from applying the $F^{14}C$ value of the tissue sample to the atmospheric bomb-pulse curve. The physical age is thus an estimate of the average time since metabolism ceased, or the turnover rate of carbon in the tissue, or a combination of both these effects. Furthermore, the physical age is the average for the whole sample fraction analyzed, which may consist of several different building parts. Data of atmospheric CO_2 are from the CaliBomb homepage (http://calib.org/CALIBomb/, accessed November 3, 2020) with the Levin data set (15-17). This curve is valid for the northern hemisphere, with insignificant regional variations (37).

TABLE 1 Clinical Characteristics of the Study Cohort (N = 52)

Age, y	74 (66-81)
Male	35 (67)
Smoking	
Current smokers	15 (29)
Past smokers[a]	18 (35)
Nonsmokers	19 (36)
Diabetes	20 (38)
Hypertension[b]	43 (83)
Lipid-lowering treatment	28 (54)
Degree of stenosis, %	85 (80-95)
Body mass index, kg/m^2	27 (24-29)
Fasting lipoproteins, mmol/L	
Cholesterol	4.5 (3.5-5.2)
Low-density lipoprotein	2.7 (2.1-3.7)
High-density lipoprotein	1.0 (0.8-1.3)
Triglycerides	1.4 (1-1.8)

Values are n (%) or median (interquartile range). [a]Not actively smoking for >6 months before surgery. [b]Systolic blood pressure of >140 mm Hg or antihypertension treatment.

METHODS

A detailed description of the methods is provided in the Supplemental Material.

CLINICAL SAMPLES. In the study, 52 human atherosclerotic plaques obtained by carotid endarterectomy between 2000 and 2013 were analyzed (Table 1). One additional plaque and a fatty streak from another donor were used to study differences in physical age by comparing one of the first stages of atherosclerosis versus advanced-stage atherosclerosis. All patients, preoperatively assessed by a neurologist, had >70% stenosis and experienced symptoms (transient ischemic attack, stroke, or amaurosis fugax) or had >80% stenosis without cerebrovascular symptoms. The stenosis degree was determined according to the velocity criteria, as assessed by ultrasonography (11). Blood samples and clinical information were collected the day before surgery. The study was approved by the local ethical committee at Lund University and followed the Declaration of Helsinki. All patients gave written informed consent.

SAMPLE PROCESSING. Plaques were snap-frozen in liquid nitrogen immediately after endarterectomy. Two consecutive 1-mm-thick sections of the most stenotic region were cut from each sample, 1 for ^{14}C analysis and 1 for histologic analysis. The 1-mm section used for ^{14}C assessment was dissected macroscopically into the 3 major plaque regions: cap, core, and interface to media. ^{14}C was then assessed in each region separately and then also combined to obtain the median plaque age. The remainder of the plaque was homogenized in 13 of the 52 plaques to assess active caspase-3.

^{14}C MEASUREMENTS. Plaque samples were dried and prepared as previously described (3). The ^{14}C content of isolated plaque fragments was quantified by accelerator mass spectrometry (AMS) using the 250-keV single-stage AMS facility (Lund University) (12). Before the measurements, the carbon was extracted from the samples and converted into elemental carbon (graphite). This was achieved in a 2-step process involving the conversion of the carbon in the sample to CO_2, followed by reduction of the CO_2 to graphite. The graphite from each sample was pressed into a sample holder and placed in the ion source of the single-stage AMS facility, together with standard samples of known activity and background samples (^{14}C-free) processed in the same way. The results from the AMS measurements were converted to calendar years using CaliBomb software (13) and the Levin data set representative for Europe (Figure 1) (14-17). The age of the plaque was then determined using the operation date as a reference.

HISTOLOGIC AND IMMUNOHISTOCHEMICAL ANALYSIS. Plaque fragments were cryosectioned in transversal 8-μm sections; fixed with Histochoice (Amresco); and stained for Masson's trichrome (fibrous tissue), Oil Red O (neutral lipids), CD68 (macrophages), and alpha-actin (SMCs).

To visualize apoptotic cells in plaques, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling (TUNEL) In Situ Cell Death Detection Kit horse-radish peroxidase (POD) (Roche Applied Science) was used, according to the manufacturer's instructions. Proliferation was assessed by staining with mouse monoclonal (PC10) antihuman proliferating cell nuclear antigen (PCNA) proliferation marker (ab29) (1:100, Abcam; overnight incubation at 4 °C). Sections were subsequently incubated with biotinylated polyclonal rabbit antimouse F(ab)2 (E0413,1:200, DakoCytomation) for 30 minutes and then with peroxidase-labeled streptavidin (Vectastain ABC-AP kit, Vector Laboratories).

Measurements of the area of plaque (percent area) for the lipids, macrophages, SMCs, proliferation, and core region were quantified blindly using Biopix Q, version 2.1.8, after scanning with ScanScope Console, version 8.2 (LRI Imaging AB). TUNEL (percentage apoptotic cells of total cell count) and total cell counts were counted from the TUNEL-stained sections using QuPath, version 0.1.2.

IMMUNOFLUORESCENCE STAINING OF CD68, ALPHA-ACTIN, AND TUNEL. To assess which cells

were most commonly TUNEL^{+}, CD68 and alpha-actin were used for double staining. Sections were fixed in 4% formalin, washed in phosphate-buffered saline (PBS) and permeabilized using TritonX-100 (on ice) and then stained for TUNEL according to the manufacturer's instructions (In Situ Cell Detection Kit, TMR red, Roche, 12 156 792 910, lot: 30967500). Finally, sections were blocked using 10% bovine serum albumin. To stain for CD68, the primary antibody monoclonal rabbit anti CD68 (Cell Signaling, number 76437, lot: 1, 0.24 μg/mL) and the secondary antibody polyclonal goat antirabbit Alexa 488 (Abcam, ab 150077, 2 μg/mL) were used. To stain for alpha-actin, the primary antibody monoclonal mouse antihuman smooth muscle actin (Dako M0851, lot: 20052340, 0.089 μg/mL) and the secondary antibody polyclonal goat antimouse Alexa 488 (Abcam, ab 150077, 2 μg/mL) were used. Next, sections were washed in PBS, blocked using 0.03% Sudan black (in 70% ethanol), and washed again in PBS before being mounted using Vectashield Mounting medium for immunofluorescence with 4′,6-diamidino-2-phenylindole (DAPI) (Vector, H1200).

ACTIVE CASPASE-3 ASSESSMENT. To assess total active caspase-3 in the human carotid plaques, the remaining plaque tissue (after removing the 2 sections for histology and ^{14}C) was weighed and homogenized. Active caspase-3 was then assessed in the plaque tissue homogenate using an active caspase-3 enzyme-linked immunosorbent assay (Invitrogen Caspase-3 [active] Human ELISA, Invitrogen) and normalized against total plaque weight. The procedure was performed according to the manufacturer's instructions and analyzed with a Luminex 100 IS 2.3.

VALIDATION OF THE TUNEL STAINING. To validate TUNEL labeling of apoptotic cells in vitro, human coronary artery SMCs were used, and apoptosis was induced by tert-butyl hydroperoxide. The TUNEL staining was performed using the In Situ Cell Death Detection Kit (POD Applied Science). The TUNEL labeling was also validated using human tonsil.

IN SITU VALIDATION OF THE TUNEL STAINING ON TONSIL TISSUE. In addition to the in vitro validation of the TUNEL staining, paraffin-embedded human tonsil sections were stained with the same TUNEL kit (In Situ Cell Death Detection Kit POD, Roche Applied Science) according to the manufacturer's instructions.

STATISTICAL ANALYSIS. Histologic variables and plaque age data were log2-transformed to perform correlation and regression analyses. The log2 transformation was used to make the data less skewed and easier to understand in terms of biologically relevance. Values are presented as the median and 25th and 75th percentiles (interquartile range [IQR]) or mean and SD, depending on the distribution of the variables (normal or not). Two-group comparisons were performed using the chi-square test, Student's *t-test*, or Mann-Whitney *U* test, depending on the variable type and distribution. Similarly, Pearson's correlation test or Spearman's rho test was used for correlation analyses. Linear regression models were used to determine which histologic variables were associated with plaque age. Differences were considered statistically significant when $P < 0.05$. The Benjamini-Hochberg test for false discovery rate was used when adjusting for multiple comparisons (q value <0.05). SPSS 21 (SPSS Inc) and GraphPad 8 were used.

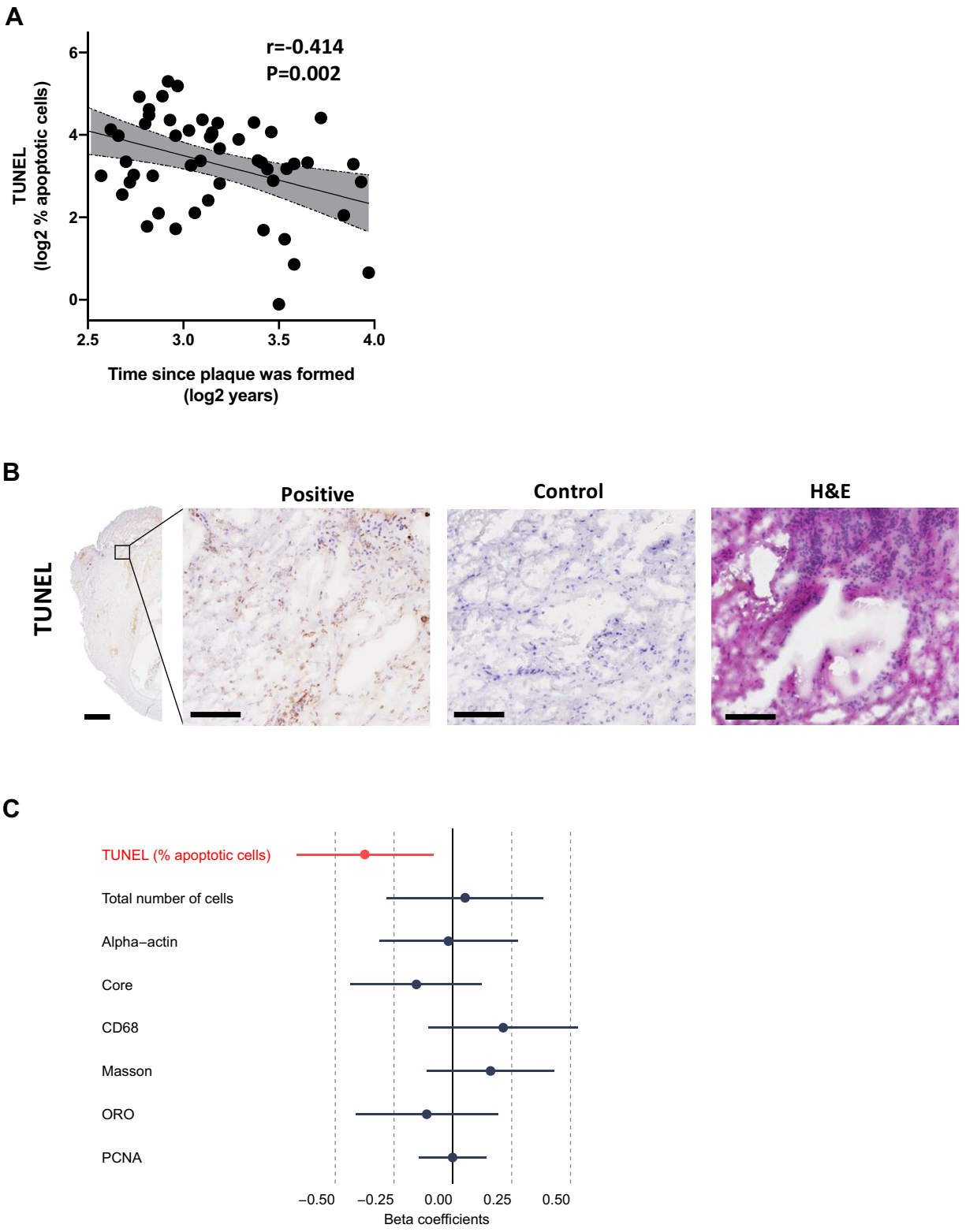
AVAILABILITY OF DATA AND MATERIALS. The data sets generated and/or analyzed during the current study are not publicly available because of the sensitive nature of the data; requests to access the data set from qualified researchers trained in human subject confidentiality protocols may be sent to Prof Gonçalves at Lund University.

RESULTS

MORE APOPTOSIS IS ASSOCIATED WITH YOUNGER PHYSICAL PLAQUE AGE. Using the ^{14}C bomb-pulse dating method, we determined the physical age of whole plaques and specific plaque regions from 52 living donors. The median age of all plaques was 8.7 years (Q1-Q3: 7.1-10.9).

In parallel, we measured the major plaque components in detail to assess the ongoing biological processes in the plaque: core size and lipids (Oil Red O staining), macrophages (CD68), SMCs (alpha-actin), fibrous tissue (Masson's trichrome), total cell count and apoptosis (TUNEL), and cell proliferation (PCNA). From all of these biological components, only a higher level of apoptosis (percentage of cells) correlated significantly with a young physical age of the plaque (r = -0.41; P = 0.002; adjusted q = 0.016 according to Benjamini-Hochberg) (Figures 2A and 2B). There were no significant associations between PCNA, CD68, alpha-actin, core size, lipids, fibrous tissue, total cell count, and physical plaque age. This indicates that these other components are not major contributors to rapid plaque progression but, rather, the degree of apoptosis. Neither did we observe any correlations between TUNEL staining and the other biological components assessed. Afterward, we performed a multiple linear regression model which showed that apoptosis (percentage of cells) was significantly associated with plaque age (P = 0.016)

FIGURE 2 TUNEL and Immunohistochemical Analysis of ^{14}C-Dated Human Carotid Plaques



(A) Apoptosis (log2 TUNEL+ cells % of all cells) is inversely correlated with the physical plaque age assessed by ^{14}C analysis (n = 52). **(B)** Representative images of human carotid plaques stained with TUNEL (nuclear staining) as well as hematoxylin and eosin. Control, an appropriate isotype control. Positive, TUNEL^{+} cells in dark brown. Scale bars: 200 μm **(left)** and 50 μm **(center and right)**. **(C)** Forest plot of beta coefficients, with 95% CIs, obtained from multiple linear regression analysis, with plaque age (log2 calendar year) as the dependent variable. Significant variables ($P < 0.05$) are highlighted in **red**. H and E = hematoxylin and eosin; ORO = Oil Red O; PCNA = proliferating cell nuclear antigen; TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling.

TABLE 2 Clinical Characteristics of the Patients (N = 52) With Physically Young (N = 26) Compared to Physically Old Plaques (N = 26) as Divided by the Median Plaque Age (≤8.7 Years or >8.7 Years, Respectively)

	Patients With Physically Young Plaques	Patients With Physically Old Plaques
Age, years	73 (66-81)	75 (67-80)
Male	19 (73)	16 (62)
Preoperative symptoms	21 (81)	17 (65)
Smoking		
Current smokers	7 (26)	8 (31)
Past smokers	8 (31)	10 (38)
Nonsmokers[a]	11 (42)	8 (31)
Diabetes	10 (38)	10 (38)
Hypertension[b]	23 (88)	20 (77)
Lipid-lowering treatment	16 (62)	12 (46)
Degree of stenosis, %	88 (75-95)	85 (80-95)
Body mass index, kg/m^2	29 (26-31)	25 (22-28)[c]
Fasting lipoproteins, mmol/L		
Cholesterol	4.6 (4.0-5.7)	4.3 (3.3-5.2)
Low-density lipoprotein	2.8 (2.2-3.9)	2.7 (1.9-3.4)
High-density lipoprotein	0.9 (0.8-1.2)	1.0 (0.9-1.4)
Triglycerides	1.5 (1.2-1.9)	1.4 (0.8-1.7)

Values are median (interquartile range) or n (%). [a]Not actively smoking for >6 months before surgery. [b]Systolic blood pressure of >140 mm Hg or antihypertension treatment. [c]Significant differences comparing physically young and old plaques are shown as $P < 0.05$; chi-square test and Mann-Whitney *U* test were used for comparisons.

(Figure 2C) even after adjusting for the other assessed biological components. This indicates that apoptosis per se is associated with rapid plaque progression.

To assess if the physical plaque age differs between early vascular changes (fatty streak) and advanced plaques, we studied these 2 from 1 single donor. Interestingly, in these 2 sections removed in 2020, we could see that the turnover in the fatty streak is much slower and that the physical age was clearly higher compared to the advanced lesion. The calculated age since formation was approximately 22 years in the fatty streak, whereas the time since formation varied between 10 and 2 years in the different regions of the advanced plaque (Supplemental Figure 1).

APOPTOSIS, PARTICULARLY IN THE CORE, IS ASSOCIATED WITH FAST-PROGRESSING PLAQUES. To compare the properties of plaques of different physical ages, we classified the plaques into physically young (≤8.7 years: n = 26) or old (>8.7 years: n = 26) based on the median plaque age. (Clinical characteristics of individuals with these plaques are presented in Table 2.) The median plaque age among physically young plaques was 7.1 years (Q1-Q3: 6.6-7.8), and the median plaque age among physically old plaques was 11 years (Q1-Q3: 9.1-12.1). Importantly, there was no significant difference when the age of the patients, plaque weight, or degree of stenosis was compared in the 2 groups.

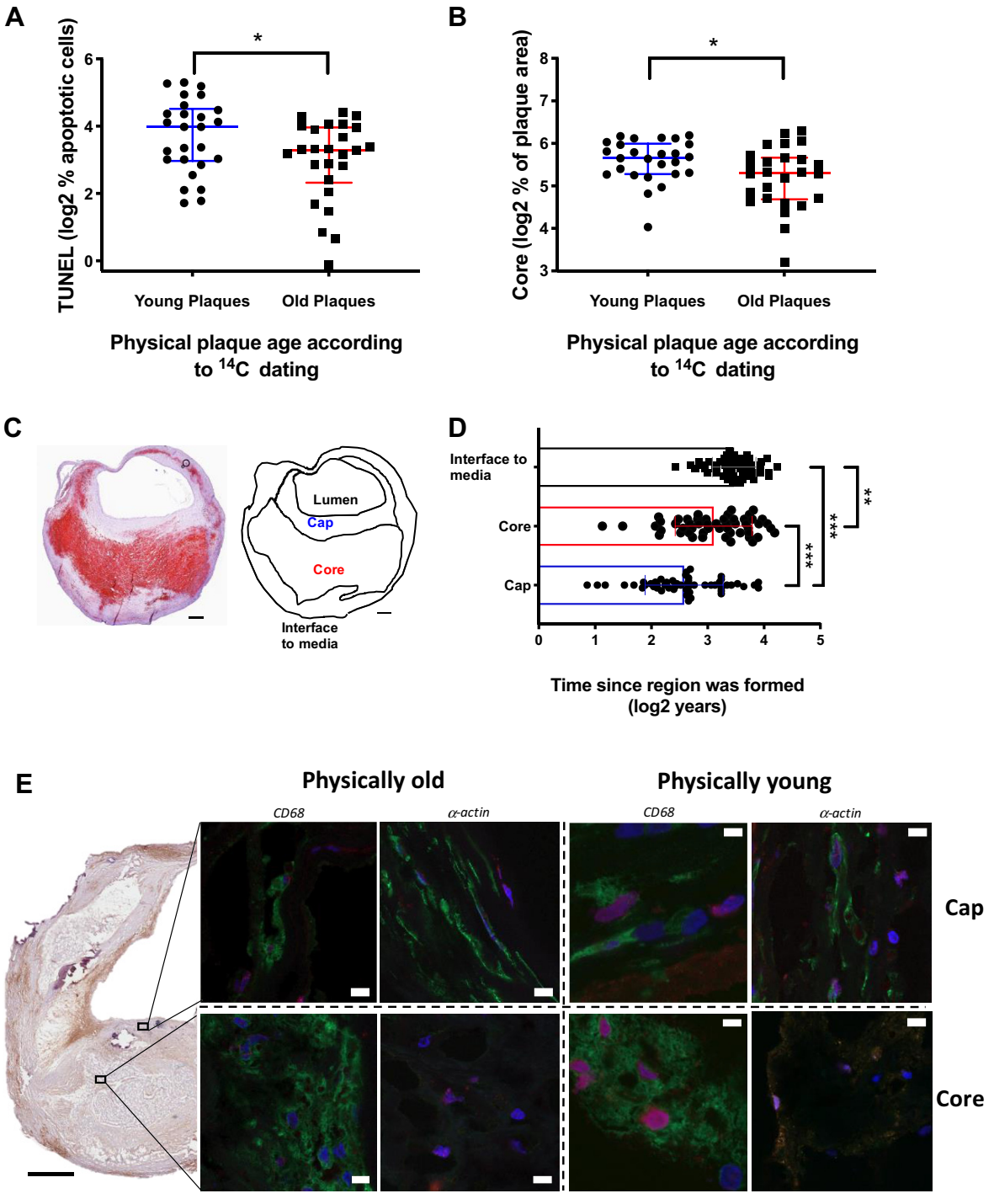
When comparing physically young with physically old plaques, only TUNEL positivity (young vs old: 3.7 [SD: 1.1] vs 3.0 [SD: 1.2] log2 % apoptotic cells; $P = 0.028$) and core size (young vs old: 5.7 [Q1-Q3: 5.3-6.0] vs 5.3 [Q1-Q3: 4.7-5.7] log2 % of cells; $P = 0.035$) (Figures 3A and 3B) were significantly different between the 2 groups. Both of these parameters were higher in young plaques, implying that apoptosis and core size are associated with fast plaque progression.

Then, we analyzed the physical age of 3 specific plaque regions (cap, core, and interface to media, dissected by a single operator) (Figure 3C) and confirmed that the cap is the most recently formed part of human atherosclerotic plaques (Figure 3D). Next, we wondered if there was an association between the composition of each region and the physical age of each region. The analysis showed that the core regions of young plaques had a greater percentage of apoptotic cells than the core regions of older plaques (young vs old: 3.9 ± 1.4 vs 3.05 ± 1.4, log2 % apoptotic cells; $P = 0.037$), whereas no other associations were significant.

APOPTOSIS AFFECTS MAJOR CELL SUBSETS SUCH AS MACROPHAGES AND SMCs. Considering the mentioned results emphasizing the importance of apoptosis, we next aimed to identify which cell types commonly undergo apoptosis and to localize them within the plaques using immunofluorescent staining. Here, we focused on macrophages (CD68$^+$) and vascular SMCs (alpha-actin$^+$). The majority of cells undergoing apoptosis were identified as alpha-actin$^+$ (SMC) and CD68$^+$ (macrophages) cells (Figure 3E). Apoptotic CD68$^+$ cells were predominantly located in the core region, and apoptotic alpha-actin$^+$ cells were predominantly located in the cap region, as expected for these cell types. This observation suggests that apoptosis affects various cells, independent of type and location.

VALIDATION OF THE TUNEL STAINING. Because apoptosis turned out to be such a relevant process for the physical age of the plaque tissue, we wanted to ascertain the accuracy of the TUNEL staining. For that, we assessed active caspase-3 levels in whole plaque tissue homogenates in a subgroup of 13 plaques. Importantly, caspase-3 levels were strongly associated with apoptosis indicated by TUNEL ($r = 0.68$; $P = 0.010$). Additionally, we verified the accuracy of TUNEL staining in tonsil tissue and in vitro using the DNA-damaging agent tert-butyl hydroperoxide (Supplemental Figures 2A and 2B). These analyses confirmed the veracity of the apoptosis assessment in plaques and further strengthened our results.

FIGURE 3 Major Plaque Features Associated With the Inferred Physical Plaque Age



Young plaques have **(A)** more apoptotic cells (TUNEL, log2 % apoptotic cells) and **(B)** larger cores (log2 % of plaque area) than old (cutoff: 8.7 years; $*P < 0.05$). **(C)** To compare the physical age between plaque regions, plaques were dissected into the cap, core, and interface region between the cap or core toward the media, and the age of each region was determined by ^{14}C-dating. Neutral lipids (Oil Red O) are shown in **red**. **Scale bars: 500 μm. (D)** The cap region is physically the youngest. **Lines and bars** represent the median and interquartile range. Kruskal-Wallis and Mann-Whiney *U* tests were used (N = 52). $**P < 0.01$; $***P < 0.001$. **(E)** Representative immunofluorescent images of TUNEL **(red)**, CD68 **(green)**, DAPI **(blue)**, and alpha-actin **(green)**. TUNEL$^+$alpha-actin$^+$ cells in the cap and TUNEL$^+$CD68$^+$ in the core region are shown. **Scale bars: 1 mm** (**left**, alpha-actin) and 10 μm (immunofluorescent). TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling.

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 6, NO. 9/10, 2021
SEPTEMBER/OCTOBER 2021:734–745

PLAQUE AGE IS INDEPENDENT OF SPECIFIC CLINICAL RISK FACTORS. Finally, we asked whether the physical age of the plaque was associated with any specific, previously known clinical risk factor. We did not identify any such association when considering the whole plaque area or the individual regions—namely, the cap, core, or interface to media. (**Figure 3C**). When comparing the clinical characteristics between patients with physically young and old plaques, only body mass index was significantly higher in the group of patients with physically young plaques (29 [Q1-Q3: 26-31] vs 25 [Q1-Q3: 22-28] kg/m^2; $P = 0.041$) (**Table 2**). Physical plaque age did not correlate to the age of the patients. Furthermore, clinical parameters such as the circulating levels of high-density lipoprotein levels, triglycerides, low-density lipoprotein levels, patient age, or body mass index were not associated with plaque apoptosis. Neither did we identify any significant differences in the physical plaque age when comparing symptomatic and asymptomatic plaques (Supplemental Figure 3).

DISCUSSION

In recent years, a growing body of evidence obtained from imaging studies has provided evidence for differences in the timeframe of atherosclerotic plaque progression (7,18,19). Based on these clinical imaging studies, rapidly progressing plaques are considered more likely to cause events. Therefore, it is a great clinical unmet need to image or target the biological processes underlying a rapid plaque progression. Moreover, no human studies have been able yet to assess the main biological determinant for the progression in situ. Here, using the novel ^{14}C bomb-pulse dating method, we identified apoptosis and core as the 2 main determinants for a rapid plaque progression in human atherosclerosis.

THE PHYSICAL AGE OF HUMAN ATHEROSCLEROTIC PLAQUES REVEALS DIFFERENCES IN PROGRESSION. Plaques have been considered to develop slowly, often over decades, but recent intravascular ultrasonography, angiography, and magnetic resonance studies have shown that the timeframe of plaque progression differs (6,7,20,21). This knowledge is of great clinical importance because fast-progressing plaques are more likely to cause symptoms. Studies performed on coronary artery plaques, including the PROSPECT (Providing Regional Observations to Study Predictors of Events in the Coronary Tree) study and the Dynamic Registry of the National Heart, Lung, and Blood Institute study, have shown that plaque progression differs over time and that plaques with a more rapid progression are more commonly associated with symptomatic disease (22-25). In addition, in a study by Yokoya et al (18), where patients underwent 4 serial coronary angiographies in a year, the most rapidly progressing plaques were associated with acute events, whereas plaques with an intermediate progression were associated with chronic obstructive events. These findings suggest that there are underlying biological differences in rapid- and slow-progressing plaques and that these differences will underlie the likelihood of rapidly progressing plaques to cause events.

In the present study, we showed that the median plaque age was 8.7 years (Q1-Q3: 7.1-10.9), which is in agreement with our previous findings (the only previous study aiming to date human atherosclerotic plaques), but now in a 5-times-larger study population (3). Importantly, the physical age of the plaque was not associated with the plaque weight, age of the patients, or degree of stenosis when comparing patients with physically younger to physically older plaques. This shows that the plaques we identified as physically young have been formed in a narrower time window and should be considered to be rapidly progressing.

Taken together, our study provides biological in situ support to previous imaging studies showing that plaques progress at various speeds, being fast or slow progressing. However, no human studies have assessed the main biological determinant for this progress.

APOPTOSIS IS THE MAIN BIOLOGICAL DETERMINANT FOR FAST PLAQUE PROGRESSION. When comparing all major plaque components histologically, apoptosis emerged as an important mechanism related to the biologic age of the plaques. Apoptosis was the only plaque component inversely correlated to the physical age of the plaques and was also the biological process with the greatest impact on the physical plaque age (according to the regression analysis, even after adjustments to the other covariates). These findings imply that apoptosis per se contributes to a more rapid plaque progression.

To compare differences in physically young and old plaques, plaques were divided by the median plaque age into 2 groups. Importantly, in support of the inverse correlation between plaque age and apoptosis, apoptosis and core size were the only components separating physically young plaques.

Core size is considered to be one of the plaque components possible to assess by several imaging

techniques and has been implicated in plaque progression. However, a growing core itself is likely not the driving factor for plaque progression, but rather the result of biological processes such as increased apoptosis, impaired efferocytosis or intraplaque hemorrhage (26,27).

Apoptosis itself is challenging to image. However, in a recent study, Chaudhry et al (28) suggested that molecular imaging of apoptosis in atherosclerosis is possible by targeting cell membrane phospholipids. By imaging phosphatidylethanolamine with radiolabeled duramycin in an atherosclerotic rabbit model, they were able to visualize lipid-rich areas with accumulated apoptotic cells. Importantly, in combination with our study, this opens up yet a new imaging possibility that could potentially be used to improve and guide future diagnostic strategies to identify and image fast-progressing plaques by assessing apoptosis/core.

RAPIDLY PROGRESSING PLAQUES HAVE APOPTOSIS OF VARIOUS CELLS IN THE CORE AND CAP. According to our study, apoptotic cells were identified to be predominantly macrophages ($CD68^+$) in the core and vascular SMCs ($alpha\text{-}actin^+$) in the cap region. This implies that apoptosis as a process regulating plaque progression is a global process in the lesion, unrestricted to a certain cell type or location.

Even though it has not been properly studied in humans previously, it has been suggested that increased apoptosis may contribute to core size and thereby also to plaque progression (29,30). Although the presence of apoptotic cells in the plaque is well described, the role of apoptosis is a matter of dispute (29). For example, SMC apoptosis has been suggested to contribute to plaque vulnerability, because SMCs play a key role in the formation of the protective cap (31). On the other hand, the role of macrophage apoptosis varies depending on the disease stage. According to a previous study be Gautier et al, apoptosis-resistant macrophages contributed to plaque formation in the early stages of atherosclerosis, whereas increased macrophage apoptosis contributed to plaque progression in more advanced disease stages (29,30,32,33). It has been speculated that in the early stages of disease, apoptosis is involved in decreasing inflammation, whereas in the later stages, possibly due to impaired efferocytosis, leads to increasing secondary necrosis and contributing to core formation (29,33). However, even though studies regarding the role of apoptosis in animal models are inconclusive, apoptosis of both macrophages and vascular smooth muscle cells are considered to be a major contributor to necrotic core formation in human atherosclerotic disease (29).

STUDY LIMITATIONS. Observed changes in the physical plaque age, as assessed by ^{14}C bomb-pulse dating, could be caused by different diets because carbon mainly enters the human body through the diet, and the ^{14}C content of different diets varies (34,35). Also, a resolution of <1 year is not to be expected. Consequently, if plaque composition changes within a timeframe below this cutoff, as reported (14,36), the relevant changes might not be detected. The typical uncertainty in a ^{14}C measurement is 0.5% to 1%, which is mainly based on the number of atoms that have been counted. An additional limitation could be that cells with high metabolism could have increased carbon turnover. Nevertheless, no such association between the assessed cells and physical age was observed.

Of note, all plaques in the present study corresponded to late-stage disease, with high degree of stenosis used as a criterion for surgical treatment. Hence, no conclusions regarding apoptosis in the early phase of atherosclerosis can be drawn. Nonetheless, patients with atherosclerosis are, in the vast majority of cases, identified in advanced stages of the disease, when advanced plaques often have already been formed. However, to detect the high-risk individuals, among patients with atherosclerosis, in order to intensify medical treatment in a personalized manner, is still a great challenge. The identification of biological components that could be used either to image such plaques or as therapeutic targets to halt the progression would, therefore, be of substantial clinical importance.

CONCLUSIONS

In the current study we showed for the first time, to our knowledge, in living patients with advanced atherosclerosis that the timeframe of plaque progression is specifically associated with apoptosis and that fast-progressing plaques have more apoptosis and a larger core size. This provides biological insight to human plaque progression and points to the potential importance of apoptosis and core formation in plaque progression. This insight could guide future diagnostic strategies, pointing out apoptosis and core as important features, to image fast-progressing plaques and/or to use as therapeutic targets to halt plaque progression.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Rapidly progressing atherosclerotic plaques are at higher risk to cause clinical complications. According to our ^{14}C bomb-pulse dating results, plaque cell apoptosis is the most relevant process contributing to a rapidly progressing plaque.

TRANSLATIONAL OUTLOOK: The identification of apoptosis as a key process for rapid plaque progression could be used to identify patients with high risk for a rapid plaque progression or as a therapeutic target to halt plaque progression.

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APPENDIX For an expanded Methods section and supplemental figures, please see the online version of this paper.