IMAGING of INTRACRANIAL ARTERIAL CALCIFICATION

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Imaging of intracranial arterial calcification
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IMAGING of INTRACRANIAL ARTERIAL CALCIFICATION

Beeldvorming van intracraniële arteriële calcificatie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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ingevolge het besluit van het college van promoties
in het openbaar te verdedigen op
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door

Remko Kockelkoren
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Prof. dr. P.A. de Jong

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Contents

CHAPTER 1

General Introduction

Stroke is, after ischemic heart disease, the most common cause of cardiovascular disease (CVD)-related mortality, accounting worldwide for more than 8 million annual deaths in a recent estimate.(1) Though stroke and cerebrovascular disease incidence numbers are slowly decreasing, it remains the second most common cause of (permanent) disability trough impaired motor function/paralysis, cognitive decline and dementia.(1–4)

A major independent risk factor for stroke and cerebrovascular disease is intracranial carotid artery calcification (ICAC) (**Figure 1**, red line), which was found to play a role in the occurrence of 75% of all strokes in a large population based study.(3) Already in 1965, when conventional x-ray examinations were the only available method to image high density structures, these 'ectopic' calcifications were examined to determine their clinical importance.(5) With the development of the computed tomography (CT) imaging technique, the sensitivity to detect and measure ICAC has greatly increased, enabling a more thorough investigation of its relevance in disease. Since then, strong relations with stroke and other cerebrovascular diseases such as lacunar infarctions, white matter disease and cognitive decline/dementia have been established.(2,3,6,7)

In addition to ICAC, intracranial calcifications can be found in most of the other intracranial arteries such as the basilar and smaller penetrating arteries as well as in smaller arteries in the basal ganglia, hippocampus (**Figure 1**, blue line) and choroid plexus. ICAC is one of the most prevalent forms of intracranial arterial calcification, occurring in over 80% of patients over 65 years of age and has shown a strong relation to cerebrovascular disease.(8,9)

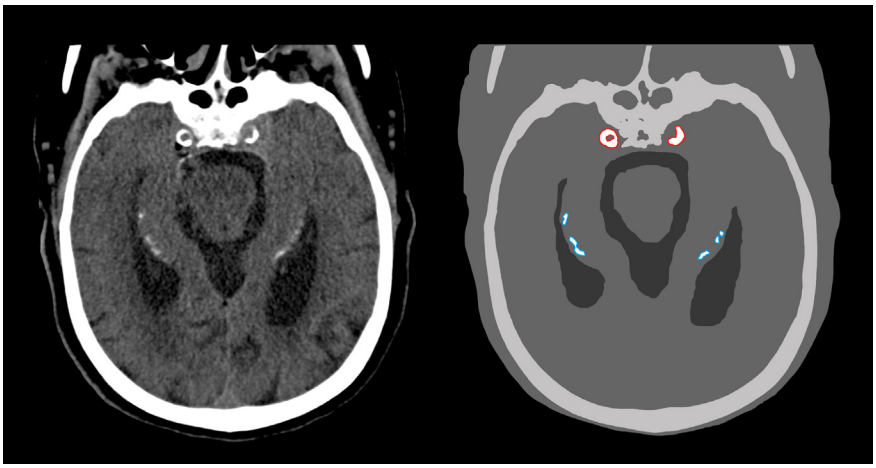

Figure 1 - Intracranial carotid artery and hippocampal calcification. Calcification of both the intracranial carotid artery (red line) and the hippocampus (blue line) on an axial CT image (left) and schematic image (right) of a 76-year-old male.

Multiple risk factors have been identified for ICAC, of which aging is consistently the strongest (age per year for severe ICAC, OR: 1.11).(10) Other, modifiable, risk factors, such as diabetes, hypercholesterolemia and smoking were also shown to have a strong relation to ICAC, however, these results are not consistent between the various available studies.(3,11,12) A possible explanation for this could be heterogeneity of these arterial calcifications, showing differences in underlying pathological mechanisms and/or etiology.

Arterial calcification on CT is often seen as a proxy of atherosclerosis, a chronic process related to classic cardiovascular risk factors that involves lipid deposition and low-grade inflammation of the arterial intima.(13,14) In atherosclerosis, fatty streaks and plaques form in the arterial wall starting with an accumulation of monocytes, macrophages and lipids.(15) In later stages of atherosclerosis, calcium(-hydroxyapatite) deposits are seen in plaques that form through a process of cell apoptosis and osteochondrogenesis.(16) Calcified plaques have been related to both plaque rupture and plaque stability, remaining a constant topic of discussion. Most likely early calcifications can make a plaque unstable, while more severe calcifications nearly always stabilize a plaque.(17)

While most of the calcifications seen on CT in the coronary arteries and probably most large extracranial arteries are atherosclerotic, a recent histopathology study revealed that ICAC is predominantly non-atherosclerotic.(18) In the study, ICAC was mainly found around the internal elastic lamina and in the arterial medial layer, both considered as medial calcification.(19) Medial calcification was first described in 1903 by Mönckeberg and is distinct from other arterial calcification in that it is also reported in the absence of atherosclerosis.(20) Medial calcification is thought to have multiple overlapping risk factors with atherosclerosis and intimal calcification such as higher age and altered glucose metabolism in diabetes mellitus however, other classic cardiovascular risk factors such as dyslipidemia and hypertension appear to play no, or a much smaller role.(21,22) Furthermore, these separate forms of calcification are thought to have a different effect on the vasculature. Whereas intimal calcification can grow intraluminal, narrowing the lumen and causing thromboembolic events through plaque ruptures, medial calcifications have a more chronic effect by decreasing elasticity and arterial remodeling leading to increased pulse pressure and damage to small vessels.(19,23) However, insight into the various risk factors between both types of calcification and differences in physiopathology is scarce due to the lack of tools to differentiate both types in vivo.

Apart from affecting the large intracranial arteries, calcifications can also be located deeper into arterioles of the cerebral vasculature. It is not uncommon to identify calcifications in the basal ganglia, pineal gland or choroid plexus on CT images. Only more recently intracranial calcifications were visualized on CT images in the hippocampus (Figure 1, blue line). Calcification of the hippocampal arteries were earlier described by histopathology, most often occurring in the hippocampal tail and body, and have been related to neuronal loss and atrophy in the areas perfused by the calcified arteries.(24) It is currently unclear whether the CT detected calcifications are the same as those described in histology. On CT images, uni- and bilateral hippocampal calcifications were present in up to 20% of people over 50 years of age and were located in similar areas within the hippocampal structure.(25) The hippocampus plays a pivotal role in transition of short- to long-term memory and spatial memory. Its role in spatial memory was previously exemplified in a study on London taxi drivers, who showed hypertrophy of the hippocampal tail compared to a control group.(26) Moreover, atrophy of the hippocampus, as seen in Alzheimer's disease, has been related to cognitive decline and dementia.(27) Hippocampal calcification might influence cognition in a similar negative way. However, because of the limited research on this subject it is unclear whether atrophy and hippocampal calcification represent a similar underlying process and whether hippocampal calcification relates to disease.

Thesis aim and outline

Current research on ICAC is mostly focused on elderly patients with cerebrovascular disease while little is known about the occurrence of ICAC in young and healthy patients. Insight in the occurrence and the longitudinal disease course may help in the set-up of early detection programs and may increase knowledge on therapeutic and preventive interventions. In these examinations, the heterogeneity of ICAC should be taken into account. For this in-vivo distinction of the dominant type of calcification, either intima or media, through imaging would be necessary. This could subsequently be used to determine the differences and overlap in underlying risk factors and, more importantly, the underlying relation to stroke and other cerebrovascular diseases such as dementia.

The implications of hippocampal calcification are less well understood and require further examination. If the calcifications found on CT and histopathology are shown to be the same disease process, they could be examined in vivo by using CT as a novel image marker for hippocampal atrophy and possible subsequent cognitive decline. Histological verification of these CT markers can therefor play a pivotal role in confirming the arterial origin and the type of calcification.

The aim of this thesis is to increase the understanding of intracranial calcifications by comparing CT findings with underlying histopathology, establish prevalence and risk factors and investigate the potential of alternative imaging methods.

In this thesis, calcification of the intracranial carotid artery (**part 1**) and hippocampus (**part 2**) are examined using CT and other modalities, compared to histopathology and prevalence and risk factors are determined.

Intracranial Carotid Artery Calcification

As a start, to gain insight into the ICAC burden in the general population and its relation to age and gender, in **Chapter 2**, we determined the prevalence and severity of ICAC in a large cohort of female and male trauma patients ranging from 0 to 100 years of age.

In **Chapter 3**, we developed a visual score that determines whether ICAC is predominantly intimal or medial by comparing CT data with histopathology in the same patients. This score is used in **Chapter 4** to examine differences and/or similarities in the risk factors/determinants of intimal and non-intimal ICAC in a large cohort of cerebrovascular disease patients.

Hippocampal calcification

In **Chapter 5**, we compared hippocampal calcification as seen on CT with histopathology to determine whether the calcifications are arterial in nature and which hippocampal regions are affected.

In **Chapter 6**, we explored the possible clinical implications of hippocampal calcifications in a pilot case-control study by comparing hippocampal calcifications in memory clinic patients and controls. Determinants for hippocampal calcifications are established in **Chapter 7** in a large cohort of patients with cerebrovascular disease.

In **Chapter 8**, we tested and validated the results found in **Chapter 5** by assessing the relation between hippocampal calcification and cognitive decline in a large geriatric cohort using multiple cognitive tests.

In **Chapter 9**, we reviewed the main findings of this thesis and discuss the next steps in future research on intracranial arterial calcification.

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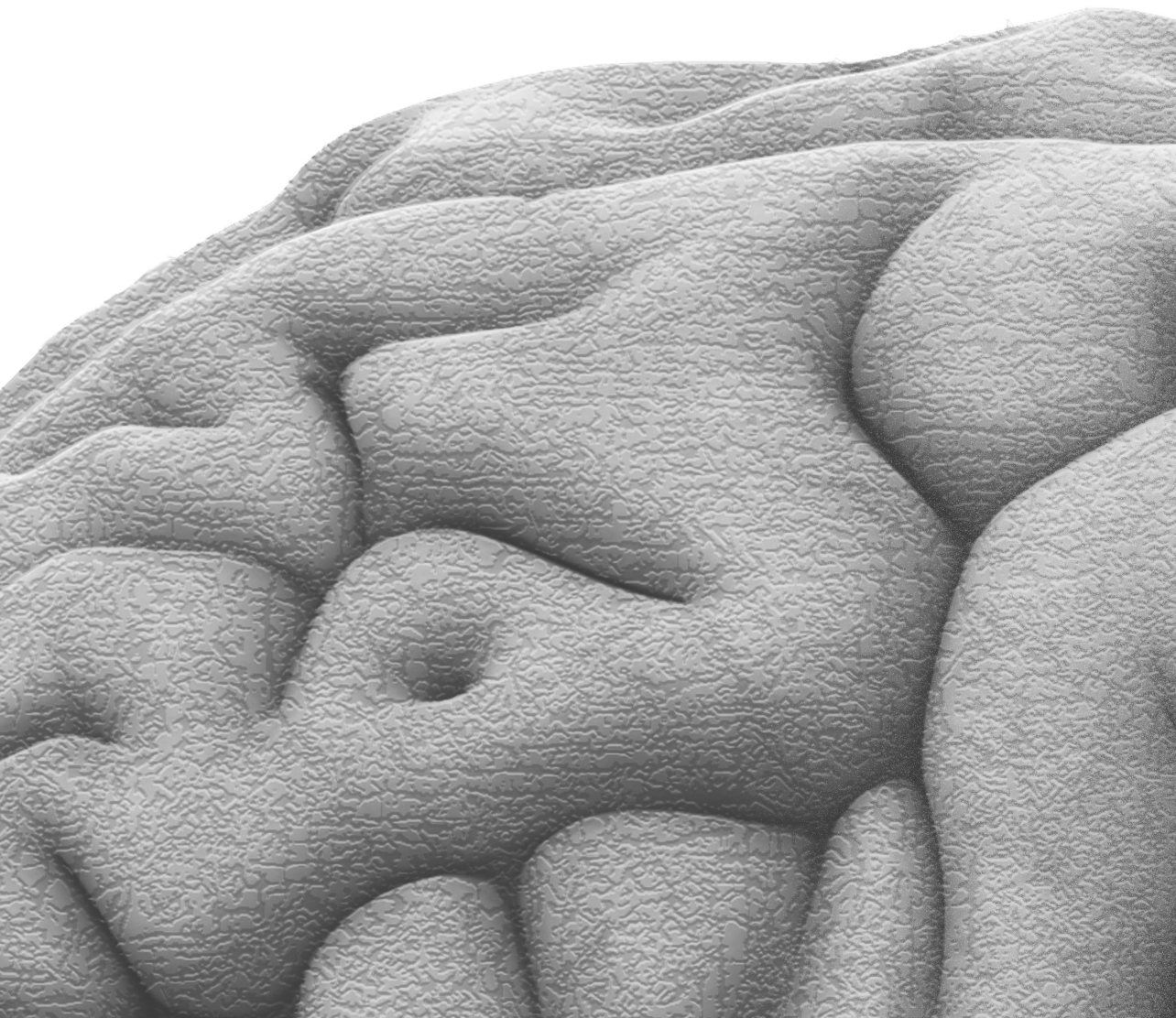
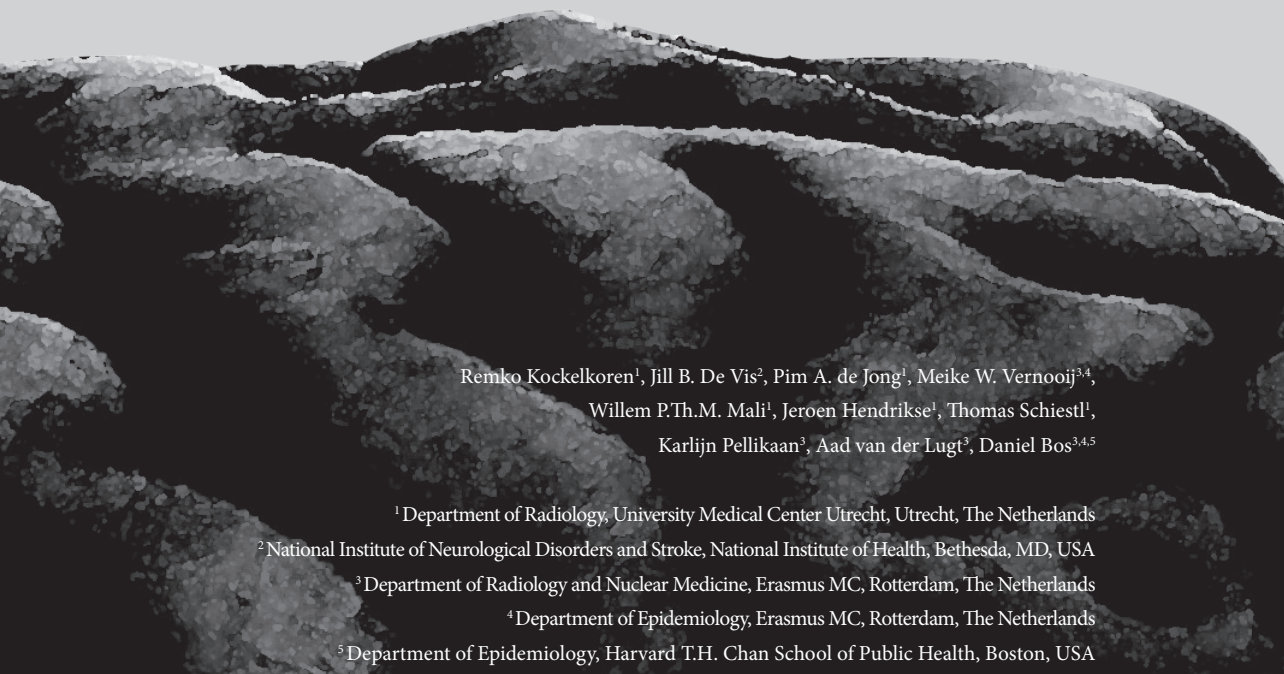


PART I

Intracranial Carotid Artery Calcification

CHAPTER 2

Prevalence and volume of intracranial carotid arteriosclerosis from infancy to old age



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Abstract

Background

Intracranial arteriosclerosis reflected in carotid artery calcification is one of the strongest risk factors for ischemic cerebrovascular disease, however, reference values from the general population are lacking and little is known about its occurrence and progression in the young. Our aim was to determine prevalence and volume of intracranial carotid artery calcification (ICAC) in a large cohort aged 0-100 years.

Methods

All patients who received a computed tomography (CT) examination because of a head trauma in two large university hospitals in the Netherlands were considered. In each center, approximately 50 women and 50 men were randomly selected per age decile (<10 years, 10-19 years etcetera), which comprised to a total of 1868 patients. ICAC prevalence and volume was visually and volumetrically assessed on unenhanced thin slice brain CT images.

Results

ICAC prevalence was 54.0% (1008/1868). ICAC was already present in 7.4% (11/148, 95% CI: 4.2-12.8%) of patients under ten years of age. Over 70 years of age ICAC was present in almost all patients. Median ICAC volume was 137mm3 (IQR: 24-407) and volume increased with age until the 10th decade of life. There was no statistically significant difference between women and men for both ICAC prevalence (p=0.61) and volume (p=0.51).

Conclusions

ICAC is already present in childhood and presence and volume increases with age in women and men in a similar fashion. Our study findings provide insight into the natural course of intracranial calcifications in the general population, which in turn can aid future studies on cerebrovascular disease

Introduction

Intracranial carotid artery calcification (ICAC), as a proxy of intracranial arteriosclerosis, is one of the most important risk factors for stroke, contributing to up to 75% of all strokes.(1) Despite that ICAC shares several similarities with calcification in other vessel beds, such as the coronary arteries, distinct differences have also become evident over the past years.(2–4) Examples include differences in the risk factor profile, showing clear associations with hypercholesterolemia and diabetes whereas associations with other known risk factors such as hypertension and smoking remain inconsistent. ICAC also has a relatively high genetic contribution.(5–7) Another important aspect of ICAC is that although it is primarily positioned as an age-related disease with its peak prevalence at older ages, there is some evidence that ICAC may already be present in adolescents and children.(8) However, robust large-scale data on the prevalence and distribution of ICAC across the whole age range is lacking. The importance of further elucidating this is twofold. First, these data will provide unique insight into the natural occurrence of ICAC, which is needed to define deviation from normality and to gain insight into its pathophysiology. Second, from a public health perspective this data would be valuable to design and test the effectiveness of personalised preventive strategies for stroke directed to those with abnormal calcium scores or its associated risk factor profile. If these calcifications indeed develop earlier than coronary calcifications and represent the same pathology as in old age, stroke prevention may even be shifted to younger individuals. An example of the potential impact of arterial calcifications at a younger age is earlier and more severe presentation of coronary artery calcification and subsequent heart disease in males.(9)

Against this background, we aimed to determine the prevalence and volume of ICAC in a large cohort of 1868 persons in the age range of 0 to 100, who underwent computed tomography (CT) of the brain in the context of head trauma screening.

Methods

Cohort

This study was a collaborative effort of the University Medical Center Utrecht and Erasmus Medical Center and was approved by the institutional review board of both centers. Informed consent was waived due to the retrospective nature of the study and the large number of participants (Approval nr. 16/092 and MEC-2016-197). All patients that were admitted to the Emergency Department of the two medical centers between January 2009 and January 2016 for a CT trauma screening were considered for the current study. The choice for this population was based on

the assumption that the usually random nature of trauma provides a reasonable representation of the general population. Furthermore, the retrospective nature of the study allowed analysis of imaging data from a large group of infants and children without the burden of a CT scan and its associated radiation dose for solely scientific purposes. Patients for whom non-enhanced thin-slice CT brain images were available, were included in the study. Patients with severe intracranial haemorrhage on CT or those who were suspected of a cerebrovascular accident as the cause of the trauma, were not eligible for the study. From all included patients, approximately 50 women and 50 men per age decile (<10, 10-19 etc.) were randomly selected per centers and comprised the final study cohort.

CT examinations

Unenhanced brain CT examinations were acquired on a Philips Brilliance 64-slice or 256-slice CT scanner (Philips Healthcare, Best, The Netherlands), or a 128-slice Siemens SOMATOM Definition AS+, or Definition Edge (Siemens, Forchheim, Germany). Patients were scanned from the skull base to the vertex and scans were reconstructed with a slice thickness ranging from 0.625 to 1 mm. The unenhanced CT images were divided in equal parts and evaluated for the presence, and volume of ICAC by six experienced raters with 1 to 14 years of experience in reading (brain) CT scans. The raters were blinded to the clinical data. Images were analysed in axial, coronal, and sagittal plane in the bone window setting (Center: 300, Width: 1600) using the Philips IntelliSpace Portal 7.0, Philips iSite Enterprise (Philips Healthcare, Best, The Netherlands), and RadiAnt Dicom Viewer (Medixant, Poznan, Poland).

Assessment of ICAC

ICAC presence was first visually determined in the intracranial carotid artery (ICA) (**Figure 1**) from the horizontal segment of the petrous internal carotid artery to the top of the internal carotid artery (Internal carotid artery segment C4 to C6 according to the classification system proposed by Bouthillier).(10) Visual assessment of ICAC was previously shown to have a good reproducibility.(11) Subsequently, ICAC was quantified using a previously described semi-automated scoring method.(5,12) Briefly, regions of interest were drawn in consecutive CT-slices, with care not to include surrounding bony structures. Next, within the regions of interest, the number of pixels over 130 Hounsfield units (HU) was determined and the calcification volume (mm3) was calculated by multiplying the number of pixels, pixel-size, and the increment. From earlier studies, this method is known to have a good interrater reliability (intraclass correlation coefficient: 0.99).(13)

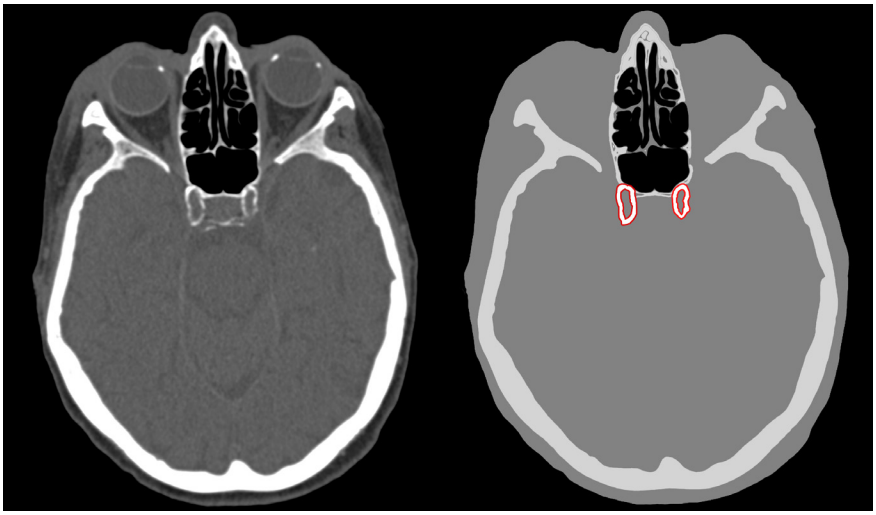

Figure 1 - Intracranial carotid artery calcification on an axial brain computed tomography scan of an 89 year old woman (left) showing severe calcifications (thick and continuous). Calcium volume is measured by manually selecting the area (red line) on all slices as shown on the schematic (right). Total calcification volume in this example was 540mm^3.

Statistical Analysis

Descriptive statistics were used to describe the characteristics of the study population; means with standard deviations and medians with interquartile ranges (Q1, Q3) for continuous variables, depending on their distribution, and counts and percentages for categorical variables. Chi-squared and Mann-Whitney U tests were used for categorical and continuous data respectively to determine differences in calcification volume between sex and age groups. Spearman's Rho was used to determine correlations in nonparametric data. The relation between ICAC presence, age per year and sex were studied in multivariable logistic regression analysis. Prevalence was determined for both the visual as well as the volumetric ICAC analysis. It should be mentioned that, due to the study design, the overall ICAC prevalence represents that of a population with a uniform age distribution.

The volumetric ICAC data was used to create an ICAC calculator which can be used by researchers and clinicians to determine a person's ICAC age- and sex-specific percentile.

Statistical significance was defined as $p<0.05$. Statistical analysis was performed using SPSS (IBM SPSS Statistics, Version 23.0. IBM Corp, Armonk, NY) and R (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/).

Results

The final study population consisted of 1868 patients (51% woman) with a median age of 49 (25-73) years, which is due to the sampling of the cohort, ranging from one month to 100 years old. Overall visual prevalence of ICAC was 54.0% (1008/1868) with a prevalence of 54.2% (512/943) and 53.6% (496/925) in women and men, respectively. Volumetric ICAC prevalence was 50.6% (945/1868) with a prevalence of 51.0 (481/943) and 50.2% (464/925) in women and men, respectively.

Age and ICAC

Calcifications were described visually as early as one year of age in men, and at three years of age in women. The first calcifications that were measured volumetrically were at six years of age in men, and seven years of age in women. ICAC prevalence, as shown in the **table 1** and **figure 2**, increased from 7.4% (95% CI: 3.8-12.9%) in the first decade of life to 100% over 90 years of age. Age was highly correlated with calcification volume (Spearman's Rho: 0.79, p<0.01) and calcification volume (Spearman's Rho: 0.82, p<0.01) with the highest mean ICAC volume occurring in patients over 90 years of age. In logistic regression analysis for ICAC prevalence age (per year) was significantly related to ICAC prevalence (OR= 1.10, 95%CI: 1.09-1.11) and sex was not (OR= 0.92, 95%CI: 0.83-1.43).

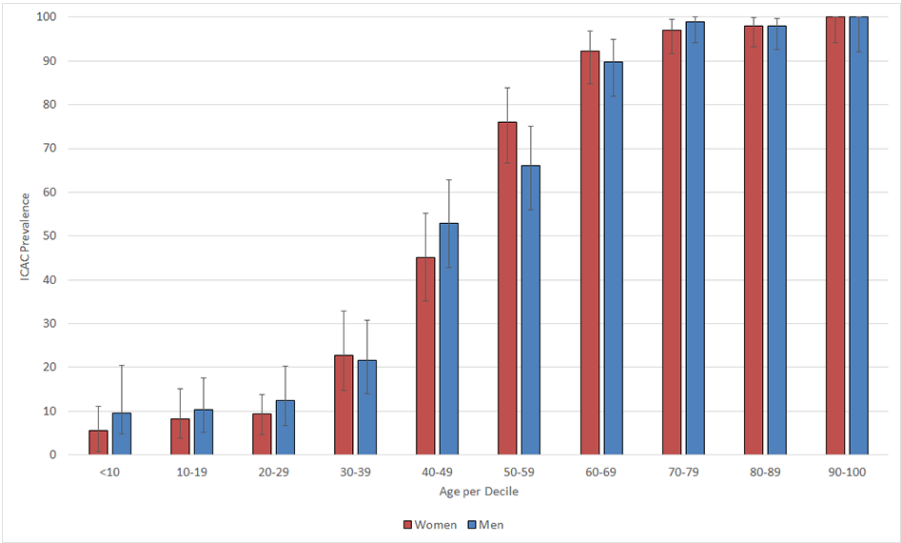

Figure 2 - Visual intracranial carotid artery calcification prevalence per decile and sex with 95% confidence intervals for the proportions. Prevalence is presented in percentages.
ICAC: Intracranial carotid artery calcification.

Table 1 - Intracranial carotid artery calcification prevalence and volume per decile and sex

Age (decile)	Women n=943			Men n=925		
	Prevalence % (Visual)	Prevalence % (Volume)	ICAC volume (Median, IQR)	Prevalence % (Visual)	Prevalence % (Volume)	ICAC volume (Median, IQR)
<10	4.0 (3/75)	3.7 (2/75)	0 (0)	10.9 (8/73)	5.4 (4/73)	0 (0)
10-19	8.3 (9/108)	4.6 (5/108)	0 (0)	10.3 (11/107)	10.3 (11/107)	0 (0)
20-29	9.4 (10/106)	6.6 (7/106)	0 (0)	12.3 (13/105)	7.6 (8/105)	0 (0)
30-39	22.8 (21/92)	14.1 (13/92)	0 (0)	21.6 (22/102)	16.7 (17/102)	0 (0)
40-49	45.1 (46/102)	41.2 (42/102)	0 (0)	52.9 (54/102)	43.1 (44/102)	0 (0)
50-59	75.9 (79/104)	70.2 (73/104)	21 (0-94)	66.0 (68/103)	61.2 (62/103)	6 (0-55)
60-69	89.0 (84/91)	86.8 (79/91)	86 (16-248)	89.7 (87/97)	88.7 (86/97)	88 (28-268)
70-79	97.1 (99/102)	97.1 (99/102)	209 (79-401)	98.9 (93/94)	97.9 (92/94)	235 (76-441)
80-89	98.0 (100/102)	98.0 (100/102)	327 (101-639)	97.9 (95/97)	97.9 (95/97)	342 (152-660)
90-100	100 (61/61)	100 (61/61)	473 (248-972)	100 (45/45)	100 (45/45)	493 (276-906)

ICAC: intracranial carotid artery calcification, IQR: interquartile range

ICAC prevalence was found to be higher using the visual measurements compared to volumetric measurements. This can be explained by the fact that multiple patients showed subtle or thin calcifications that had densities below 130 HU in which no volume could be measured. An example of evident ICAC that could not be measured volumetrically is presented in the **supplemental figure**.

Sex and ICAC

ICAC prevalence did not differ between women and men (**Figure 2**, p=0.61). The increase of prevalence over age was comparable and calcifications were found in the first decade in both women and men (Table). Also, calcification volume was not significantly different between men and women (p=0.51). ICAC volume by age and sex is shown in **figure 3**.

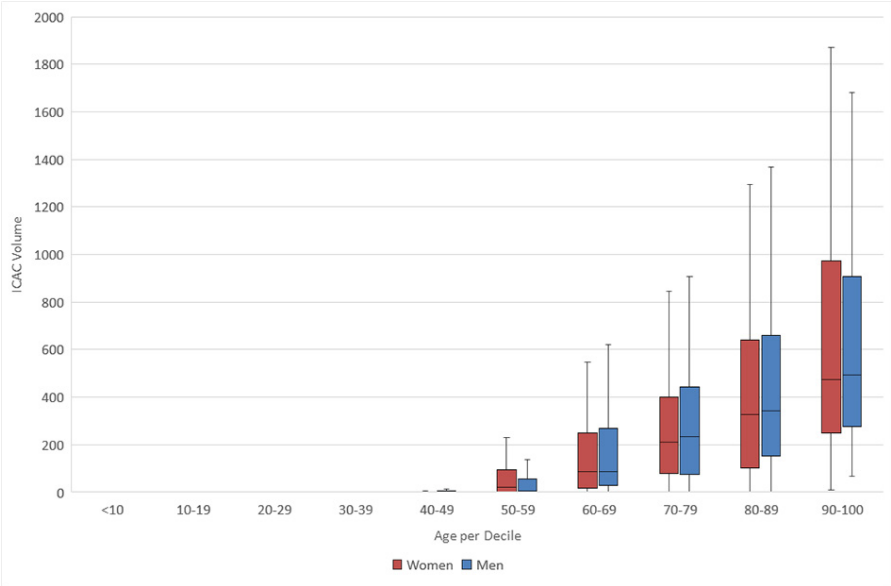

Figure 3 - Box-plots of intracranial carotid artery calcification volume (in mm3) per decile and sex. ICAC: Intracranial carotid artery calcification.

Discussion

In this large study, we determined the prevalence and volume of ICAC across the entire age range from 0-100 years. We found that ICAC already occurred in the first decade of life with a prevalence of 7.4%. From thereon prevalence steadily

increased until the 70 years of age, after which practically all persons in the study showed ICAC. Additionally, ICAC volume increased with age and was comparable between women and men across the entire age spectrum. Given the known association between ICAC and risk of cerebrovascular events, these data on the natural history may aid identification of persons at risk with larger than normal ICAC volume.

ICAC prevalence in this study was found to differ substantially from previously published prevalence estimates for calcifications in other arteries, demonstrating the heterogeneity of these radiological markers.(3) The most profound difference compared to, for example, the aorta or coronary arteries is the substantial ICAC prevalence of 7.4% in patients under the age of 10. While this is not a new finding, as ICAC has previously been reported at this age on CT and histology, it clearly deviates from the general perception of arterial calcification prevalence only starting at middle age and beyond.(14–16) Compared to the extensive imaging data on arterial calcification in the elderly, little is known about its prevalence in the general population under 40, except in rare diseases such as Generalized Arterial Calcification of Infancy (GACI).(17) While large scale imaging data on calcification prevalence in the young is unavailable, presumably because of inherent objections to CT population studies in this age group, its prevalence has been reported in histological studies. In a postmortem study in adolescents and young adults between 15 and 34 years old, Strong et al. examined first signs of atherosclerosis and arteriosclerotic calcifications by histology, which is much more sensitive than CT. They found that calcified lesions are first found in the abdominal aorta and the corresponding prevalence in 598 patients between 15 and 19 years of age was 0.5%. In comparison, in our study, ICAC prevalence on CT was 11% in the 134 patients in this age group.(18) A question that remains is whether ICAC seen at this early age represent the same pathology as in old age and might therefore be predictive for the occurrence of stroke later in live.

Several explanations may underlie the substantial age-related differences of ICAC compared to calcification in extracranial vessels. First, it was recently found that ICAC is a highly heritable trait (heritability estimate of 47%), which indicates a prominent role of genetic predisposition for ICAC.(6) This substantial genetic underpinning of ICAC may directly translate to the higher prevalence at younger ages and also might explain why ICAC is already present at this young age. This is further corroborated by the fact that traditional cardiovascular risk factors such as diabetes, hyperlipidaemia, smoking, and hypertension are generally still rare at younger ages. Second, Bergevin et al hypothesized that ICAC occurrence, specifically in children, might arise due to increased rigidity of the ICA starting

at five years of age caused by an increase in surrounding connective tissue.(14) Increased rigidity combined with the tortuous shape of the ICA results in turbulent blood flow which in turn may lead to arterial disease.(19,20) It was previously suggested that the calcifications found in the ICA of children and young adults could be in the range of normal physiology related to ageing.(8) We could imagine that these calcifications, in the range of normal physiology, are a protective mechanism to support the arterial wall of the relatively thin/fragile intracranial arteries in these young children from relatively high, and very variable, blood flow velocities causing turbulent flow.(21) Why the ICA is prone to arterial damage already in children and whether preventive efforts would be effective and desirable in this younger population below 40 years of age, should be investigated. Prospective analysis of ICAC in young patients looking into risk factors, biomechanical factors and flow analysis will be required to provide more insight on this subject.

In this study women and men showed similar ICAC prevalence and, strikingly, comparable ICAC volumes over all ages. Arterial calcifications are generally more prevalent and severe in men.(3,22,23) In the coronary arteries, men are affected earlier in life and show more severe atherosclerosis and calcifications.(9) Similar differences have not been reported for ICAC.(5) Whereas calcifications of coronary and most other arteries are primarily affected by atherosclerosis and located in the arterial intima, ICAC was mainly found to occur in/around the internal elastic lamina in both young and old people.(14,24) Calcifications found in this elastic layer have been shown to be more related to arteriosclerosis, or stiffening/hardening of the artery, than atherosclerosis.(20,25) Furthermore, internal elastic lamina calcification, often considered as a form of medial calcification, is described to be more prevalent in women in contrast to atherosclerosis, which in turn could account for the comparative prevalence and volume of ICAC between sexes.(9,20) The impact of these different types of calcification for women and men on local and distal circulation remains to be determined.

The strength of this study is the large cohort size and inclusion of approximately 100 women and men per decile in whom ICAC was measured both visually and volumetrically. Still, some limitations should be considered. First, this cohort consisted of patients who were examined in our trauma centers. This is a population with, presumably, a slightly higher (cardiovascular) disease risk profile compared to the general population. Still, we believe that this cohort is a reasonable approximation of the general population due to the fairly random nature of trauma. More importantly, for this study we utilize previously acquired CT data that would otherwise require a large group of children, (young) adults, and elderly to receive a brain CT scan, which would be ethically challenging. Second,

different CT scanners were used for the examinations in this study which could have a small influence on ICAC volume measurement. On the other hand, the use of different CT scanners could make the data representative for a higher number of imaging centers. Third, even though multiple methods to measure ICAC were used in this study, some subtle calcifications were most likely missed. We know from a previous study that the spatial resolution of CT is limited in detecting small or thin calcifications of a certain size when compared to histology.(26) Thus, the true prevalence of ICAC may actually be higher than our estimations.

Conclusions

In this cohort ICAC was found to be already prevalent in the first decade of life in both sexes and prevalence continually increased until it was present in virtually all patients over 70 years of age. Furthermore, ICAC volume showed no evident differences between women and men. Our findings provide an extensive overview of natural history of ICAC in the general population which can be used to further examine this highly prevalent arterial disease.

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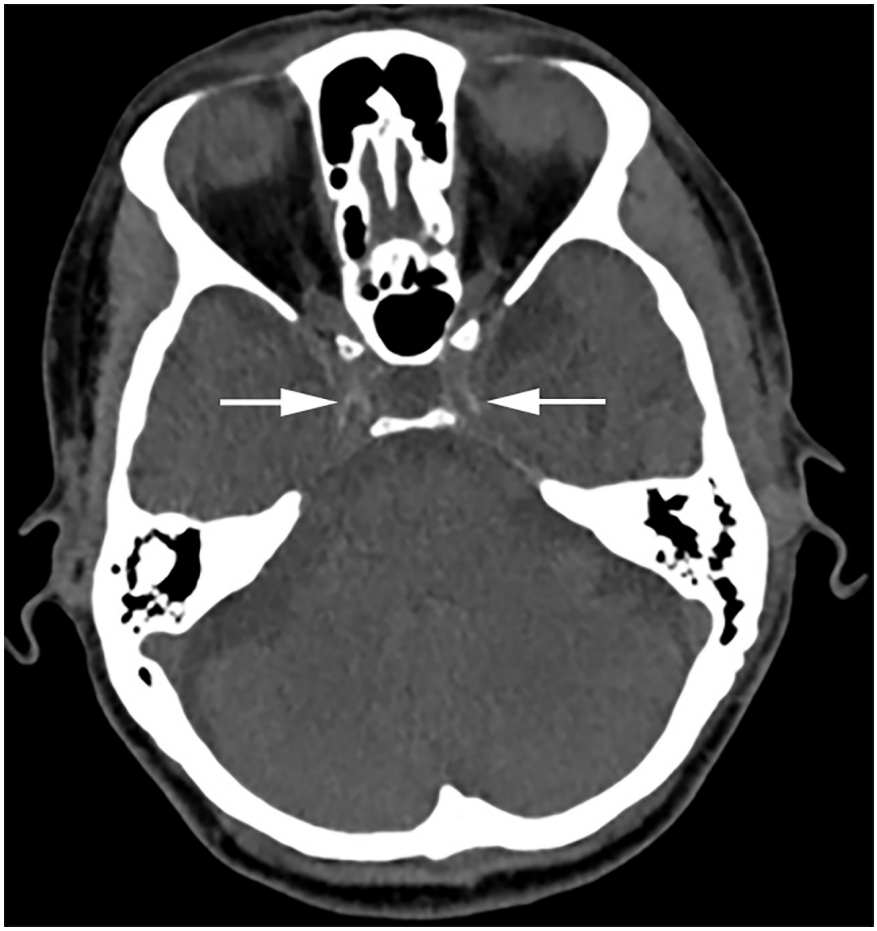
Supplementary material



Supplementary figure - Example of bilateral concentric ICAC which is not registered on volumetric analysis due to low measured density (lower than 130HU).

CHAPTER 3

Computed tomographic distinction of intimal and medial calcification in the intracranial internal carotid artery

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Abstract

Background

Intracranial internal carotid artery calcification (ICAC) is associated with stroke and is often seen as a proxy of atherosclerosis of the intima. However, it was recently shown that these calcifications are predominantly located in the tunica media and internal elastic lamina (medial calcification). Intimal and medial calcifications are thought to have a different pathogenesis and clinical consequences and can only be distinguished through ex vivo histological analysis. Therefore, our aim was to develop CT scoring method to distinguish intimal and medial ICAC in vivo.

Methods

First, in both intracranial carotid arteries (ICA) of 16 cerebral autopsy patients the intimal and/or medial calcification area was histologically assessed (142 slides). Brain CT images of these patients were matched to the corresponding histological slides to develop a CT score that determines intimal or medial calcification dominance. Second, performance of the CT score was assessed in these 16 patients. Third, reproducibility was tested in a separate cohort.

Results

First, CT features of the score were circularity (absent, dot(s), <90°, 90-270° or 270-360°), thickness (absent, ≥1.5mm, or <1.5mm), and morphology (indistinguishable, irregular/patchy or continuous). A high sum of features represented medial and a lower sum represented intimal calcifications. Second, in the 16 patients the concordance between the CT score and the dominant calcification type was reasonable. Third, the score showed good reproducibility (kappa: 0.72, proportion of agreement: 0.82) between the categories intimal, medial or absent/indistinguishable.

Conclusions

The developed CT score shows good reproducibility and can differentiate reasonably well between intimal and medial calcification dominance in the ICA, allowing for further (epidemiological) studies on ICAC.

Introduction

Intracranial carotid artery calcification (ICAC) on Computed Tomography (CT) is an independent predictor of stroke in the general white population and was associated with 75% of all stroke in the Rotterdam study.(1) Calcifications of the intracranial carotid artery (ICA), commonly referred to as the carotid siphon due to the tortuous shape, have also been associated with lacunar infarctions(2,3) and white matter hyperintensities on Magnetic Resonance Imaging (MRI).(4)

While ICAC is often seen as a proxy of atherosclerotic burden and thereby are thought to be situated in the arterial intimal layer, a recent histology study showed that these calcifications are predominantly non-atherosclerotic and are located in the tunica media and around the internal elastic lamina.(5) Because calcifications of the medial layer of the vascular wall and calcifications around the internal elastic lamina are thought to be related and are therefore from here on grouped as medial calcifications.(6)

Calcification of the arterial intimal and medial layer are presumed to have a different pathogenesis and clinical consequences.(7) The intimal layer consists of endothelial cells that 'proliferate' in the process of atherosclerosis growing into the arterial lumen forming plaques that narrow the lumen and can rupture, causing thromboembolic events.(8) Whereas the medial layer consists of smooth muscle cells and elastic fibers which have a function in regulating blood flow and arterial pressure. Calcification of the media is thought to cause stiffening, reduce compliance and limit distensibility. This can cause an increase in pulse wave velocity and pulse pressure and subsequently chronic damage to the brain tissue. (9,10) Distinction of these types of ICAC would be desirable to further investigate their respective roles in (neuro)vascular disease.

Ex vivo histological analysis is the gold standard to distinguish intimal and medial calcification.(7) Studies that differentiate both calcification types in vivo using either physiological tests (high ankle-brachial index), X-ray or ultrasound have been carried out but the literature in this field is sparse. Most studies are focused on the lower extremities and breast arteries due to technical limitations of visualizing arteries deeper in the body, especially in the head.(11,12) Hence there is no valid in vivo method to separate ICAC while the distinction between both could prove valuable.

Therefore, our aim was to develop a scoring method which distinguishes intimal from medial ICAC in vivo to enable further epidemiological studies on this subject. For this purpose, CT imaging was preferred based on its ability to visualize arterial calcifications and its wide use in clinical practice. A comparison between histology

data and CT imaging characteristics was performed per patient to develop a visual CT score which could determine intimal or medial calcification dominance in the ICA. Subsequently, performance and reproducibility of the score was determined.

Methods

Study Design

Our study consisted of three steps. In the first and second step we developed and validated the CT score in a single cohort. In the third step we assessed the reproducibility of the score in a separate cohort which will be described later. For the first two steps we included 16 consecutive deceased patients in whom cerebral autopsy was performed between April 2014 and February 2015. The inclusion criteria was a brain CT examination 6 months prior to autopsy. All patients had a routine autopsy for which consent was given by their next of kin. Permission for the evaluation of rest material from the autopsies was given by the local Biobank Review Committe, under protocol number 15-252. Material was handled in a coded way that met the criteria of the code of proper use of human tissue, used in The Netherlands. Informed consent for data used in assessing reproducibility was waived by the Medical Ethical Testing Committee (METC) due to the retrospective nature of the study (approval number: 16/092).

Histological Analysis

After removal of the brain, the ICA was dissected as close to the petrous bone as possible and removed. Subsequently the arteries were fixed in 4% formaldehyde, and (partially) decalcified using diaminoethylene tetraacetic solution (EDTA). Decalcification was necessary to maintain the morphology of the vascular wall during tissue processing, and does not influence the analyses since the matrix previously altered by the calcification process still remains.(12) The arteries were divided in a proximal (C4 and C5 according to the classification system proposed by Bouthillier) and a distal (C6) segment (**Supplemental Figure 1**),(13) the intersection was perpendicular to the lumen Per segment, 2-3 histological slides, stained with hematoxylin and eosin and elastin van Giesson, were used to digitally analyse the total surface of calcifications in the intima, in the media and around the internal elastic lamina, as previously described.(5) Calcifications were characterized by quite sharp demarcated, acellular spots and areas, which were dark pink to purple colored on hematoxylin and eosin stained slides. For this study digital images of 142 slides were available.

Dominant calcification type was determined per patient by adding the calcification areas in all slides to a summed intimal and medial calcification

burden. If the summed area of medial calcification was larger than the summed area of intimal calcification the patient was categorized by histology as medial dominant and vice versa.

CT Image Acquisition and Analysis

The 16 patients were scanned on a Philips Brilliance 64-slice or 256-slice CT scanner (Philips Healthcare, Best, The Netherlands) from the skull base to the vertex. Tube voltage was either 120kVp or 140kVp and tube current ranged between 200 – 250 mAs. For adequate detection of the subtle/thin calcifications only non-contrast enhanced CT's were used with slice thickness between 0,625 and 1mm. The image quality was assessed and all images were deemed to be of good/adequate quality without evident artefacts (beam hardening, photon starvation, noise) that could potentially influence image evaluation. Images were assessed in bone setting (Center: 300 Hounsfield Units – Width: 1600 Hounsfield Units) in all planes (axial, sagittal and coronal).

ICAC on CT was analysed in concordance with the 142 histological slides. The location of the histological slide in the ICA was registered and with this information the corresponding CT slide could be approximated by using multiplanar reconstruction in any direction. Both the histological slide and corresponding CT image were then displayed side by side to allow for adequate comparison (**Figures 1 and 2**).

Calcification Score: Development

In step 1 histological and CT slices were compared to develop a visual calcification score. For this, emphasis was put on distinctive features of intimal and medial calcification, as earlier described in literature.(9) I.e. medial calcifications have been described as being often thin and progress in a circular pattern while intimal calcifications (that co-localize with atherosclerotic plaques) are more clustered and often grow intraluminal.(14,15)

To develop the score, points were assigned to the different calcification characteristics found in the analysis of the matched CT- and histological slides. The points awarded per characteristic were weighted according to their relation to either medial or intimal calcification. Subsequently, for each combination of characteristics it was decided whether it was associated with dominant intimal or dominant medial calcifications and based on all combinations an optimal point threshold for dividing calcification dominance was determined. If a calcification was visible on CT, but the amount was too limited to assign any morphological characteristics to, it was classified as indistinguishable.

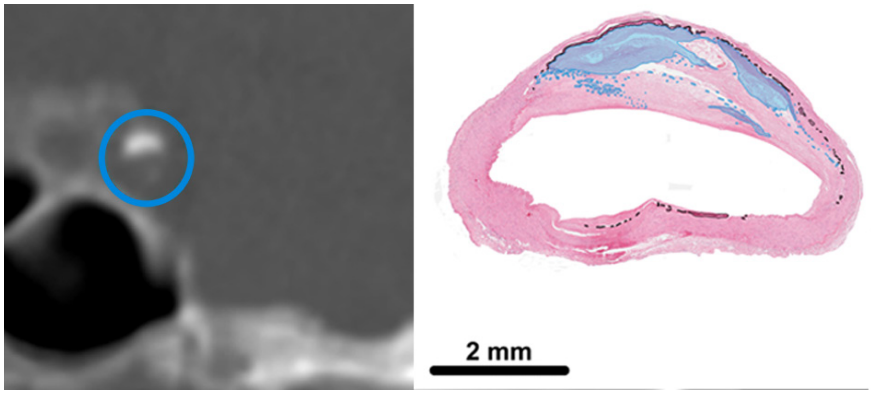

Figure 1 - Intimal calcification in the intracranial carotid artery (ICA) on a coronal brain CT image (left) and on a histological slide (right). On CT a blue circle is placed around the ICA. In histology the intimal calcification area is light blue and the calcification area of the internal elastic lamina indicated by the black line.

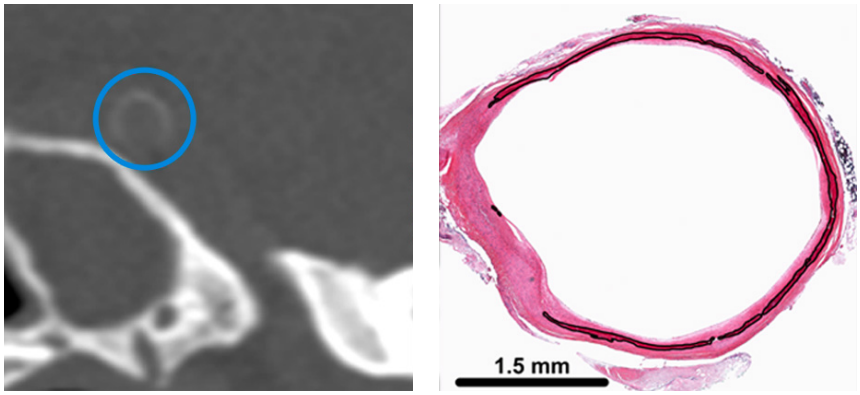

Figure 2 - Internal elastic lamina calcification in the intracranial carotid artery (ICA) on a coronal brain CT image (left) and on a histological slide (right). On CT a blue circle is placed around the ICA. Calcification area of the internal elastic lamina is indicated by the black line. Reprinted from A. Vos et al. Stroke. 2016;47:221-223 (Figure 1A) under a CC BY license, with permission of the American Heart Association, original copyright 2016 American Heart Association.

Calcification Score: Performance

In step 2 the performance of the developed calcification CT score was evaluated in the 16 autopsy patients by two raters (P.A.D.J. and J.B.D.V.) with respectively fourteen and three years of experience in reading CT scans and who were not involved in the development of the CT score. Both raters were blinded for the

histological reference standard. The final score of either dominant intimal, medial or indistinguishable/absent as rated with the CT score was compared to the results of the dominant calcification type as was determined by histology.

Calcification Score: Reproducibility

To assess the applicability of the score the interrater reliability and agreement were determined. For that purpose, a larger sample size was preferred. Therefore in step 3 we randomly selected CT scans from 48 patients who were part of a larger study of patients with a suspected acute stroke.(17) To determine applicability of the score in the general population and compare the reproducibility to (suspected) stroke patients we matched patients from an ongoing study on trauma patients by age and gender to the 48 stroke patients. Trauma patients were chosen as reference over other patient groups as they represent a random sample of the general population due to the random nature of traumas. Two raters (J.B.D.V. and R.K) with respectively three and two years of experience in reading CT scans independently scored the 96 CT scans.

Statistical Analysis

For the CT-histology performance, three by three tables were drawn and diagnostic characteristics were determined. For the interrater reliability and agreement study kappa values, with 95% confidence intervals, and proportions of agreement were calculated, respectively. Kappa's of circularity and thickness were linearly weighted as these are ordinal measures. A Kappa value of >0.60 was regarded as good reliability. Data analysis was performed with R (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/).

Results

The median age of the 16 patients (ten males, six females) was 64 years (range 44-85). Two of the patients were diagnosed with diabetes mellitus type 2 and one patient was known to have kidney disease. In 11 patients intracranial pathology was found; tumor (2), hemorrhage (5), and ischemia (4). Dominant calcification types of all histology slides are presented in **table 1**.

Table 1 - Distribution of calcification dominance per histological slide

	Location	Medial	Intimal
Left ICA n, (%)	Proximal	23 (62)	9 (24)
	Distal	25 (81)	5 (16)
Right ICA n, (%)	Proximal	34 (85)	4 (10)
	Distal	29 (85)	5 (15)
Total		111(78)	23 (16)

ICA: intracranial carotid artery, n= number, Proximal = C4-C5 and Distal = C6 (Classification of Bouthillier(14))

Development

The analysis of the matched histological and CT slices showed that, in accordance with previous literature, intimal calcifications were overall clustered, thick and scattered/patchy throughout the artery (**Figure 1**) whereas medial calcifications were overall circular, thin and continuous (**Figure 2**). Based on these findings the characteristics for the score were determined as circularity, thickness and morphology. The final visual score was as follows (**Figure 3, Table 2**):

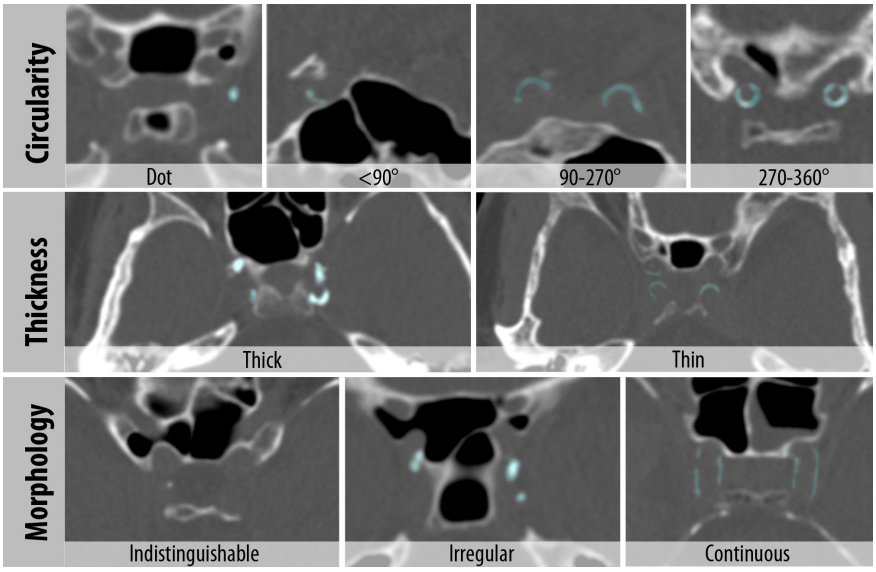

Figure 3 - Intracranial carotid artery calcification score with Circularity (Dot, <90°, 90-270° and 270-360°); Thickness (Thick ≥ 1.5mm and Thin < 1.5mm) and Morphology (Indistinguishable, Irregular, Continuous). Calcifications are highlighted (light blue). In these examples, all images are in the axial viewing plane except for the <90° and 90-270° images which are in the coronal plane.

Calcification circularity was scored from absent (0 points) to 270-360 degrees (4 points) based on the most circular part in a perpendicular viewing plane. The maximal circularity within the ICA is used for the score.

Calcification thickness was scored as either thick (1 point) or thin (3 points). If the thickest calcification exceeded 1.5mm, measured perpendicular to the arterial wall, the calcifications were scored as thick. The maximum thickness of both ICA's was used for the score.

Calcification morphology could either be indistinguishable (0 points), irregular/patchy (1 point) or continuous (4 points). Indistinguishable was used to describe dot like calcifications that were too small to assign any morphological characteristics to, and were therefore not categorizable. Irregular and/or patchy referred to spread out or irregular calcifications. Continuous calcifications were regular calcifications spread over a longer arterial segment. The dominant morphology pattern over the trajectory of the ICAs was scored.

Table 2 - Intracranial carotid artery calcification score for unenhanced CT

Characteristic		Points
Circularity	Absent	0
	Dot(s)	1
	<90 degrees	2
	90-270 degrees	3
	270-360 degrees	4
Thickness	Absent	0
	Thick ≥ 1.5mm	1
	Thin < 1.5mm	3
Morphology	Indistinguishable	0
	Irregular/Patchy	1
	Continuous	4

<7: Dominant Intimal, ≥ 7: Dominant Non-Intimal

Based on all possible combinations of points a threshold of seven was determined as the optimal value to separate intimal and medial calcifications (**Supplemental Table 1**). If after adding up the points the total was lower than seven, the calcifications were thought to be dominantly intimal, whereas if the total was over or equal to seven the calcifications were thought to be dominantly medial. An exception holds for calcifications that were deemed indistinguishable. Furthermore, the CT score determined the dominant calcification type on a patient basis instead of for each

ICA separately as on histological slides the calcification pattern was symmetrical in 88% of patients. The score was applied accordingly for determining performance and reproducibility.

Performance

Performance of the CT score can be found in **table 3**. Rater 1 classified eight patients correctly, four indistinguishable/absent and four incorrectly. Rater 2 classified nine patients correctly, three indistinguishable/absent and four incorrectly. When accounting for the indistinguishable calcifications, the performance of the score was reasonable. Three misclassified ICACs were found in both raters. The majority of incorrectly classified patients had little ICAC which made them harder to classify. Most notable was one patient in which dominant intimal calcification was scored as dominant medial calcification because of the more circular nature of the intimal calcifications in this particular patient. This was the only patient in our sample showing intimal calcification with high circularity. In the other two, dominant medial calcification was scored as dominant intimal calcification. On the CT images multiple dot-like calcifications were present whereas the medial calcification that was visible on the histological slides was too subtle to detect on CT. As another example of the detectability of these calcifications: in the three patients where both raters scored absent, medial calcifications were present that could not be detected due to a limited detection threshold of CT.

Table 3 - Performance of the calcification score in the intracranial carotid artery

		Model			
		Medial	**Intimal**	**Absent***	**Total**
Histology	**Rater 1**				
	Medial	6	2	4	12
	Intimal	2	2	0	4
	Absent	0	0	0	0
	Total	8	4	4	16
	Rater 2				
	Medial	6	3	3	12
	Intimal	1	3	0	4
	Absent	0	0	0	0
	Total	7	6	3	16

*or indistinguishable

Reproducibility

The median age of the 96 scored patients (58% male) was 69 (range 41-90) years. Results of the CT score by both readers were as follows (Supplemental Table 2 and 3). Reproducibility results can be found in Table 4. Reliability between the raters was 0.80 (CI: 0.72-0.88) for circularity, 0.75 (CI: 0.65-0.86) for thickness and 0.70 (CI: 0.57-0.82) for morphology. The proportion of agreement between the raters was 0.74 (CI: 0.64-0.82) for circularity, 0.81 (0.72-0.88) for thickness and 0.80 (0.71-0.87) for morphology. Reliability and agreement for the overall score was 0.72 (CI: 0.60-0.84) and 0.82 (CI: 0.73-0.89). Both reproducibility and agreement were comparable between the stroke and reference cohort (**Table 4**).

Table 4 - Reproducibility of the calcification score

	Full cohort (n=96)	Stroke cohort (n=48)	Reference cohort (n=48)
Interrater reliability*			
Circularity	0.80 (0.72-0.88)	0.77 (0.65-0.89)	0.82 (0.72-0.92)
Thickness	0.75 (0.65-0.86)	0.76 (0.61-0.90)	0.75 (0.60-0.91)
Morphology	0.70 (0.57-0.82)	0.65 (0.46-0.83)	0.74 (0.58-0.91)
Calcification score	0.72 (0.60-0.84)	0.71 (0.53-0.88)	0.73 (0.57-0.90)
Interrater agreement			
Circularity	0.74 (0.64-0.82)	0.73 (0.58-0.84)	0.77 (0.62-0.87)
Thickness	0.81 (0.72-0.88)	0.81 (0.67-0.91)	0.81 (0.67-0.91)
Morphology	0.80 (0.71-0.87)	0.77 (0.62-0.88)	0.83 (0.69-0.92)
Calcification score	0.82 (0.73-0.89)	0.81 (0.67-0.90)	0.83 (0.69-0.92)

*Kappa

Discussion

In this study, we showed that a reasonable distinction can be made in vivo between intimal and medial calcification dominance in the ICA. We developed a CT score that can be used on non-contrast enhanced CT images and has good agreement between raters.

ICAC on CT images in relation to intracranial pathology has been studied extensively using both visual and quantitative scores. Visual scores provide an overall good, quick and reproducible measure of ICAC burden(17) whereas quantitative or volumetric calcification measurement are time intensive but do provide a more accurate (volumetric) depiction of the calcium load/burden.(1)

However, the previous qualitative scores were not designed to separate intimal and medial calcification. The score developed in this study combines the visual characteristics circularity, thickness and morphology, as previously used in ICAC scores.(3,4,18–20) Our comparison with histology showed that these described characteristics, after some adaptation, were a good basis to separate intimal and medial calcifications.

The distinction of intimal from medial calcification could be of importance for several reasons. One, ICAC is related to stroke and other intracranial vascular diseases, but it is unclear whether this is attributable to intimal calcifications, medial calcifications or a combination of both. Also, the etiology (and ultimately treatment) of dominant intimal or medial disease may be different. Two, in case of medial calcification the compliance of the arterial wall may be diminished leading to an increased pulsatility and thereby pulse wave velocity.(21,22) Medial calcification in the ICA may therefore explain the observed associations between ICACwith white matter hyperintensities(4) and acute small vessel infarcts.(3) Three, medial calcification was found to comprise 71% of the dominant calcification burden and thereby it's influence on physiopathology may be underestimated.(5) Four, clinically medial calcification is mostly thought to be a passive and chronic process, however, in a recent review it was shown that it is actually an active process resembling bone formation.(9) The limited knowledge on the clinical consequences and therapeutic options for medial calcification mainly comes from genetic syndromes and epidemiological studies outside the brain. Our CT score provides the opportunity to further investigate the role of medial calcification in cerebrovascular diseases.

The data presented in this paper demonstrated that the majority ICAC was either scored correctly according to their dominant calcification pattern on histology, or were scored as indistinguishable or absent. When calcification was absent on CT images, it was at times still detected on histology in very minute amounts but remained under the detection threshold of CT. The indistinguishable category represents patients with too little visible calcification on CT images to be properly classified. Most likely, due to their limited amount of calcification, those patients are also the ones who least benefit from early intervention and thereby a lack of classification could be considered to be less of importance within this group of patients. The remaining 25% of our ICAs were classified incorrectly. Interestingly, the majority of these patients also showed little calcification on the CT images and thereby could have been on the edge of what is reasonable to classify. Adequate stratification of these calcifications will probably remain problematic with the current resolution of CT scanners.

A major strength of this study is that we used histology as a reference standard. Often CT scores are developed without a solid reference standard. Another strength of this study is the use of good quality CT data with thin slices (0.625 – 1 mm) and multiplanar reconstructions to evaluate the calcifications. Intracranial calcification is often scored on a slice thickness of 5mm and on axial plane only. Considering that the cavernous ICA is on average 4-7mm in diameter and often runs parallel on axial images, the number of slices suitable for scoring is limited. Also, evaluating the circumferential degree of calcification on thick axial slices can be quite problematic. For these reasons, we would also recommend future studies who are implementing the presented CT score, to evaluate thin slices and make use of multiplanar reconstructions.

There are some limitations to this study. First, even though we attempted to make an optimal comparison between histology and CT we cannot be entirely certain that the CT images exactly represented the histological slides. However, we do think that our 142 comparisons gave us overall a good impression of the morphological differences in medial and intimal calcification on CT. Second, the performance was evaluated on a limited sample size of 16 patients. The histological analysis of the calcifications is time consuming (all individual calcification deposits are manually scored) which limited us in the number of samples we were able to analyse. Third, strokes of cardioembolic origin will not be captured by the calcification score. This could influence results in analysis of ICAC and stroke etiology and should therefore always be considered. Lastly, although we did find reasonable performance in a labour intense and therefore small study and good reproducibility between two raters in a larger sample, further investigations are needed before the CT score can be applied as a diagnostic tool in patients. Nevertheless, the presented score could already be used in epidemiological and genetic studies investigating the clinical implications of medial calcification.

Conclusion

In conclusion, we developed a reproducible CT based scoring tool that can reasonably distinguish intimal and medial calcification dominance in the intracranial internal carotid artery. This CT score could be used for future in vivo epidemiological studies to better understand the role of intimal and medial calcification at this location of the arterial system. The ability to separate these different forms of calcifications and the improved phenotyping of ICA disease in vivo could help to better understand cerebrovascular diseases.

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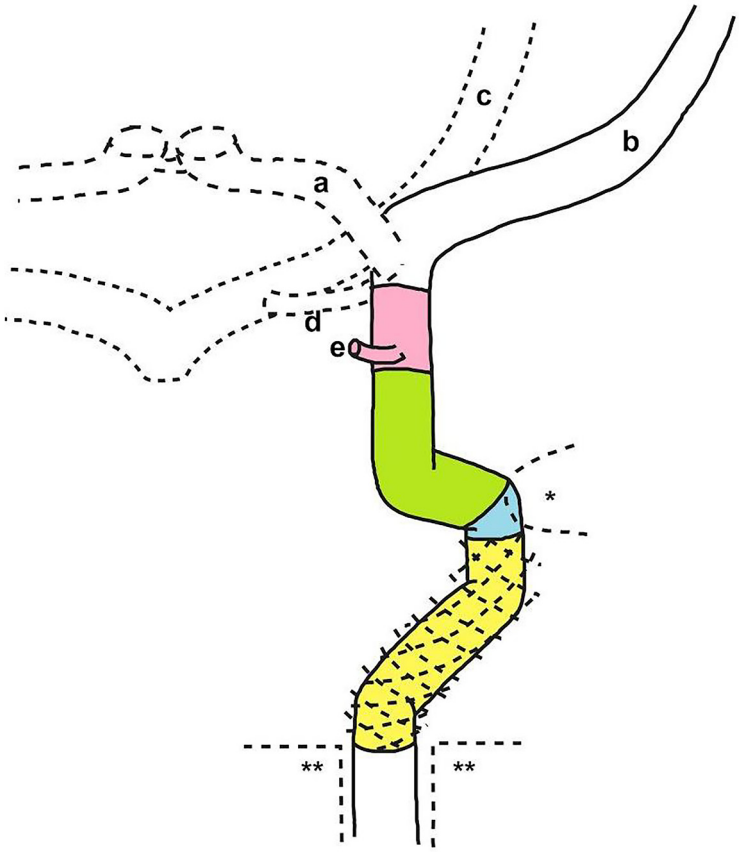
Supplemental information



Supplemental Figure 1 - Schematic of the intracranial carotid artery and subsequent cerebral arteries. Histology of calcifications was analysed per patient both proximal (C4-C5) and Distal (C6). a: Anterior cerebral artery; b: Middle cerebral artery; c: Posterior cerebral artery; d: Posterior communicating artery; e: Ophthalmic artery; Pink: C7; Green: C6; Blue: C5; Yellow: C4 (cavernous sinus); *anterior clinoid process; ** carotid canal and foramen lacerum.

Supplemental Table 1 - Combinations of calcification characteristic points and calcification association

Points	Circularity	Thickness	Morphology	Dominance
2	Dot	Thick	Indistinguishable	Intimal*
3	Dot	Thick	Irregular	
4	Dot	Thin	Indistinguishable	
5	<90°	Thick	Irregular	
	Dot	Thin	Irregular	
6	90-270°	Thick	Irregular	
	<90°	Thin	Irregular	
	270-360°	Thick	Irregular	
7	<90°	Thick	Continuous†	
8	90-270°	Thin	Irregular	Medial
	270-360°	Thin	Irregular	
9	90-270°	Thick	Continuous	
	<90°	Thin	Continuous	
	270-360°	Thick	Continuous	
10	90-270°	Thin	Continuous	
11	270-360°	Thin	Continuous	

= Associated with intimal calcification
= Associated with medial calcification
= Unassociated

*Except for indistinguishable calcifications, † Did not occur in our CT images.

Supplemental Table 2 - Calcification score results in 48 patients with suspected stroke

		Rater 1		
		Medial	**Intimal**	**Absent***
Rater 2	Medial	18	2	0
	Intimal	5	12	0
	Absent*	1	1	9

*or indistinguishable

Supplemental Table 3 - Calcification score results in 48 trauma patients

		Rater 1		
		Medial	**Intimal**	**Absent***
Rater 2	**Medial**	21	0	0
	Intimal	6	8	0
	Absent*	0	2	11

*or indistinguishable

CHAPTER 4

Risk factors for atherosclerotic and medial arterial calcification of the intracranial internal carotid artery

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Minor revisions at Atherosclerosis

Abstract

Background

Intracranial carotid artery calcification (ICAC) is an important risk factor for stroke. The calcifications can occur both in the intimal and medial layer of the vascular wall. The aim of this study is to assess whether medial calcification in the ICA is differently related to risk factors for cardiovascular disease, compared to intimal calcification.

Methods

Unenhanced thin slice computed tomography (CT) scans from 1132 patients from the Dutch acute stroke study cohort were assessed for dominant localization of calcification (medial or intimal), by one of three observers based on established methodology. Associations between known cardiovascular risk factors (age, gender, body mass index, pulse pressure, eGFR, smoking, hypertension, diabetes mellitus, hyperlipidemia, previous vascular disease, and family history) and the dominant localization of calcifications were assessed via logistic regression analysis.

Results

In the 1132 patients (57% males, mean age 67.4 years [SD 13.8]) dominant intimal calcification was present in 30.9% and dominant medial calcification in 46.9%. In 10.5% no calcification was seen. Age, pulse pressure and family history were risk factors for both types of calcification. Multivariably adjusted risk factors for dominant intimal calcification only were smoking (OR 2.09 [CI 1.27-3.44]) and hypertension (OR 2.09 [CI 1.29-3.40]) and for dominant medial calcification diabetes mellitus (OR 2.39 [CI 1.11-5.14]) and previous vascular disease (OR 2.20 [CI 1.30-3.75]).

Conclusions

Risk factors are differently related to the dominant localizations of calcifications, a finding that supports the hypothesis that the intimal and medial calcification represent a distinct etiology.

Introduction

Intracranial carotid artery calcification (ICAC) is an important independent risk factor for stroke in the general population. These calcifications are often interpreted as a proxy for atherosclerosis. However, already in 1965 it was described that calcifications in the siphon of the carotid artery are not only found in the intimal layer of the vascular wall, but also in the medial layer and around the internal elastic lamina.(2) Recently it was shown that ICAC is predominantly located around the internal elastic lamina.(3) Calcifications in this area are considered to be medial arterial calcifications.(4)

Medial calcifications have been described in multiple arteries, including femoral and breast arteries.(5,6) Breast arterial calcifications (BAC), as visualized on mammography, are thought to be exclusively medial. (5) BAC has a similar incidence in patients with angiographically normal arteries and patients with coronary heart disease.(7) However, the incidence of BAC was found to be higher in patients with an indication for coronary angiography than in the general population.(7) Therefore, is has been hypothesized that BAC shares some, but not all risk factors for atherosclerosis.(7)

Combining previous literature, we know that there is a strong association between ICAC and stroke, and that ICAC is predominantly medial. Furthermore, it is hypothesized that risk factors for medial arterial calcifications can be partly different from risk factors for atherosclerotic vascular disease. Therefore, it is important to determine what risk factors influence the different types of ICAC. If medial arterial calcifications are indeed an important factor in the development of stroke, differences in risk factors could influence current clinical practice regarding risk reduction.

Previous reports described associations between ICAC and age, diabetes, hypercholesterolemia, hypertension, smoking, history of cardiovascular disease and high white blood cell count.(8-12) However, these studies did not take the different localizations of calcification in the vascular wall into account. Based on a comparison with histopathology, we recently described a computed tomography (CT) scoring system that can determine the dominant calcification type in the intracranial carotid artery (ICA).(13) This scoring system allows us to evaluate the effect of risk factors on the different dominant calcification types. The aim the current study is to assess whether medial calcification in the ICA is differently related to risk factors for cardiovascular disease, compared to intimal calcification.

Methods

Cohort

The patients were derived from the DUtch acute Stroke Study (DUST) cohort; a multi-center cohort study of 1393 patients with suspected acute ischemic stroke. Patients were included if the following criteria were met: 1) older than 18 years, 2) National Institutes of Health Stroke Scale ≥2, or 1 if an indication for intravenous thrombolysis with recombinant tissue type plasminogen activator was present, 3) acute neurological deficit of less than nine hours of duration. Patients were excluded from the study if another diagnosis on admission non-contrast Computed Tomography (CT) explained the neurological deficits, and in case of a known contrast allergy or previously known renal failure at the time of admission. At the time of admission patient characteristics were collected, including blood pressure, height, weight, previous vascular disease (including previous diagnosis of myocardial infarction, transient ischemic attack, stroke and peripheral vascular disease or previous vascular intervention), smoking, and family history of vascular diseases (1 or more first degree relative <60 years). Furthermore, laboratory tests, including serum creatinine and glucose, and a non-contrast CT-scan were performed. DUST was approved by the Medical Ethical Committee of the participating hospitals under protocol number 08-373. Informed consent was obtained from all patients for use of the data.(14)

CT imaging

Multiple CT scanners were used in the participating centers. The number of detectors ranged from 40 to 320 (LightSpeed VCT, GE Healthcare, Milwaukee, Wisconsin; Brilliance 40, Brilliance 64, and Brilliance iCT 256, Philips Healthcare, Best, the Netherlands; Sensation 64, Siemens, Erlangen, Germany; Aquilion ONE, Toshiba Medical Systems, Tokyo, Japan) at 120 kV and 300-375mAs. Patients were scanned from the skull base to the vertex and scans were reconstructed with a slice thickness ranging from 0.625 to 1 mm.

CT scoring

For all patients the presence, morphologic characteristics and severity of ICAC was scored on the thin slice CT data by one of three readers with at least 2 years of experience reading CT images. (PdJ, JdV, RK) The agreement between the readers was previously found to be good, with kappa's ranging from 0.70-0.80. The readers were blinded to the clinical data. Using the previously developed scoring model points were awarded for different morphologic aspects of the calcifications (0-4 points for circularity, 0-3 points for thickness of calcifications, and 0-4 points for

continuity of calcification over a longer arterial segment). Based on the total score (range 0-11 points) the calcifications were defined as dominantly intimal (score <7 points), dominantly medial (score ≥7 points), indistinguishable (continuity of calcification unclassifiable, due to the presence of only very small amounts of calcification), or absent. (**Figure 1**; 13) Furthermore the severity of the calcifications was scored in a four-tier system (none, mild, moderate, severe) as previously described by Woodcock and colleagues. (15)

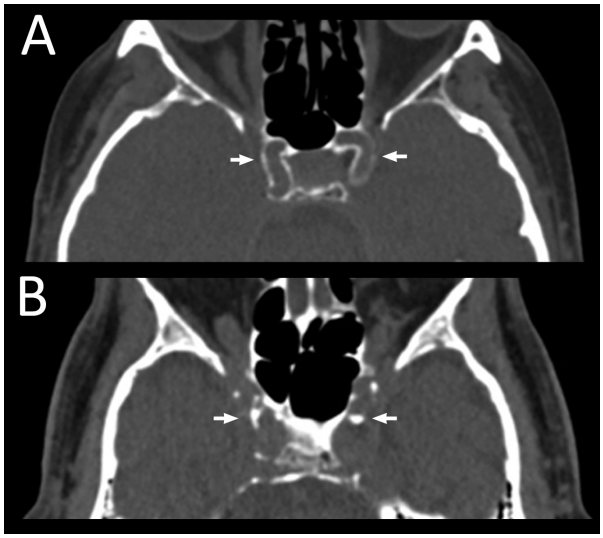

Figure 1 - Examples of predominant intimal and predominant medial calcification on CT A. An example of predominant medial calcification: a thin continuous line of calcification (arrows). B. An example of predominant intima calcification: thick dots of calcification (arrows).

Clinical and laboratory characteristics

Body mass index (BMI) was calculated using the collected weight and height of the patients. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.(16) This formula calculates the eGFR based on gender, age, serum creatinine and race. Since information regarding race was not available in the dataset, we calculated the eGFR as if we only included white patients. Given the localization of the study, we assumed the majority of patients to be white. We also calculated the eGFR as if all patients were black (multiply by 1.159) and repeated the analysis, to see the possible influence of these increased eGFR values on the results.

Statistical analysis

Characteristics (age, gender, body mass index, systolic blood pressure, diastolic blood pressure, pulse pressure, serum creatinine, eGFR <60 mL/min/1.73m^2, glucose, smoking, hypertension, diabetes mellitus, hyperlipidemia, previous vascular disease, and family history) were expressed according to the location of calcifications. Continuous normally distributed variables were presented as mean and standard deviation, skewed continuous variables as median and interquartile range. Categorical variables were presented as percentages.

The crude and adjusted associations between the risk factors and the dominant localization of calcifications (intimal or medial) were assessed by logistic regression. We first compared patients with calcifications that could be classified as dominant intimal or dominant medial by binomial logistic regression. We repeated this analysis in patients with severe, often more easily classifiably, calcifications. Finally, as comparing only patients with calcifications can mask a protective effect of a determinant and hide a risk factor that is significant for both types of calcification, we also conducted multinomial logistic regression for assessing risk factors for a dominant intimal and a dominant medial pattern of calcifications in all patients suspected for acute stroke (with indistinguishable calcifications as reference category).

Results are presented as odds ratios (OR) with 95% confidence intervals (CI). Adjusted OR were adjusted for all other risk factors listed. Missing values in variables used in multivariable analyses were accounted for via multiple imputation using the fully conditional specification Markov Chain Monte Carlo (MCMC) method. Multivariable analyses were run on 15 imputed datasets and combined using Rubin's rule. Statistical analyses were conducted using SPSS Statistics version 21.0 (IBM Corporation, New York, United States). P-values of <0.05 were considered significant.

Results

In all 1393 patients included in the study a CT-scan was performed. However, in 261 of these 1393 patients thin slice unenhanced CT-images were not available. Therefore ICAC was scored in the remaining 1132 patients (57% males, mean age 67.4 years [SD 13.8]). In 30.9% (350 of 1132) of patients a dominant intimal pattern of calcification was found, in 46.9% (531 of 1132) a dominant media pattern. In 11.7% (132 of 1132) of patients the main localization of calcification could not be determined (indistinguishable category) and in 10.5% (119 of 1132) calcifications were absent. (**Table 1**) Calcifications were severe in 34.5%, moderate in 28.1% and mild in 26.9%. (**Supplemental table I**)

Table 1 - Characteristics in association with dominant localization of calcifications

	Absent n=119	Intima n=350	Media n=531	Indistinguishable n=132
Age (years)	48.6 ± 10.9	67.4 ± 10.8	73.8 ± 11.2	58.5 ± 12.3
Gender (male)	66 (55.5%)	228 (65.1%)	269 (50.7%)	80 (60.6%)
Body mass index (kg/m^2)	26.3 (24.7-30.6)	26.5 (23.8-29.3)	26.0 (23.1-28.4)	26.6 (24.4-29.2)
Pulse pressure (mmHg)	60.7 ± 16.6	72.8 ± 22.2	76.0 ± 25.0	64.5 ± 21.3
eGFR <60 mL/min/1.73m^2	5 (4.2%)	38 (11%)	90 (17.1%)	11 (8.4%)
Current smoker	40 (34.2%)	124 (37.6%)	102 (21.2%)	38 (30.6%)
Hypertension	33 (28.0%)	205 (58.7%)	313 (59.6%)	44 (33.8%)
Diabetes mellitus	6 (5%)	53 (15.1%)	102 (19.3%)	10 (7.6%)
Hyperlipidemia	17 (14.4%)	141 (41.5%)	192 (37.4%)	38 (29.5%)
Previous vascular disease	28 (23.5%)	165 (47.1%)	281 (52.9%)	39 (29.5%)
Family history (positive)	37 (36.6%)	83 (33.2%)	92 (27.5%)	24 (22.2%)
Severity of calcification				
Absent	119 (100%)	0 (0%)	0 (0%)	0 (0%)
Mild	0 (0%)	102 (29.1%)	71 (13.4%)	132 (100%)
Moderate	0 (0%)	159 (45.4%)	159 (29.9%)	0 (0%)
Severe	0 (0%)	89 (25.4%)	301 (56.7%)	0 (0%)

Variables described as mean ± standard deviation for continuous variables, median (interquartile range) for skewed continuous variables, and number (%) for categorical variables.

Variables described as mean ± standard deviation for continuous variables, median (interquartile range) for skewed continuous variables, and number (%) for categorical variables.

Dominant intima versus dominant media calcifications

In the 881 patients who were either scored as predominant media or intima calcification, logistic regression analysis showed that patients with predominant media calcification were significantly older (OR 1.49 per 10 years of age [CI 1.29-1.73]), more often female (OR male gender 0.64 [CI 0.47-0.87]), smoked less often (OR 0.57 [CI 0.40-0.80]), and more often had a history of previous vascular diseases (OR 1.49 [CI 1.06-2.09]) than patients with predominant intima calcifications (**Table 2**). The other risk factors did not differ significantly between the two groups. The results were comparable when only the patients with severe calcifications (often more easily classifiable) were analyzed by binominal regression. (**Supplemental table II**) The result did not change when using the eGFR values as if all patients were black. (**Supplemental table III**)

Table 2 - Association between risk factors and a predominant medial localized calcification pattern in patients with classifiable intracranial carotid artery calcifications*

Determinant	Crude OR (95% CI) for having predominant media calcification	P-value	Adjusted OR (95% CI) for having predominant media calcification	P-value
Gender (male)	0.549 (0.416-0.725)	<0.0005	0.640 (0.472-0.868)	0.004
Age (per 10 years)	1.680 (1.475-1.913)	<0.0005	1.491 (1.287-1.726)	<0.0005
BMI (kg/m^2)	0.974 (0.937-1.012)	0.183	0.979 (0.943-1.016)	0.265
Pulse pressure (per 10 mmHg)	1.058 (0.999-1.120)	0.054	1.007 (0.945-1.073)	0.827
eGFR <60 mL/min/1.73m^2	1.697 (1.131-2.546)	0.011	1.310 (0.844-2.034)	0.229
Current smoker	0.446 (0.326-0.609)	<0.0005	0.568 (0.402-0.801)	0.001
Hypertension	1.037 (0.787-1.363)	0.795	0.737 (0.531-1.024)	0.069
Diabetes mellitus	1.342 (0.933-1.930)	0.113	1.448 (0.965-2.172)	0.074
Hyperlipidemia	0.844 (0.638-1.117)	0.236	0.724 (0.508-1.032)	0.074
Previous vascular disease	1.302 (0.988-1.717)	0.061	1.489 (1.062-2.086)	0.021
Positive family history	0.765 (0.536-1.092)	0.140	0.984 (0.697-1.387)	0.925

eGFR indicates estimated glomerular filtration rate; BMI, body mass index; OR, odds ratio; and 95%, CI 95% confidence interval. All adjusted OR are adjusted for all other determinants listed. *classifiable calcifications: all patients with calcifications that could be scored as predominant intimal or predominant medial; patients without calcifications or indistinguishable calcifications were not included in this analysis.

Risk factors for intimal and risk factors for medial calcifications

When assessing the risk factors for dominant intimal calcification in all patients, including the patients without or with unclassifiable calcifications, by multinomial regression, intimal calcifications were associated with older age (OR 1.89 per 10 years of age [CI 1.53-2.32]), higher pulse pressure (OR 1.12 per 10 mmHg [CI 1.01-1.24]), smoking (OR 2.09 [CI 1.27-3.44]), hypertension (OR 2.09 [CI 1.29-3.40]) and a positive family history (OR 1.80 [1.08-2.99]). Medial calcifications were associated with older age (OR 2.84 per 10 years of age [CI 2.94-3.51]), higher pulse pressure (OR 1.12 per 10 mmHg [CI 1.01-1.25]), diabetes mellitus (OR 2.39 [CI 1.11-5.14]), previous vascular disease (OR 2.21 [CI 1.30-3.75]) and positive family history (OR 1.75 [1.04-2.95]). (**Table 3 and Figure 2**) The result did not change when using the eGFR values as if all patients were black. (**Supplemental table IV**)

Table 3 - Association between risk factors and a predominant intimal or predominant medial localized intracranial carotid artery calcification pattern in the full cohort

Determinant	Adjusted OR (95% CI) for having predominant intima calcification	P-value	Adjusted OR (95% CI) for having predominant media calcification	P-value
Gender (male)	1.371 (0.878-2.142)	0.166	0.860 (0.553-1.339)	0.505
Age (per 10 years)	1.886 (1.533-2.320)	<0.0005	2.837 (2.942-3.510)	<0.0005
BMI (kg/m^2)	0.983 (0.935-1.033)	0.491	0.965 (0.918-1.015)	0.169
Pulse pressure (per 10 mmHg)	1.118 (1.005-1.242)	0.040	1.122 (1.010-1.246)	0.031
eGFR <60 mL/min/1.73m^2	0.545 (0.254-1.169)	0.119	0.718 (0.344-1.498)	0.377
Current smoker	2.092 (1.273-3.438)	0.004	1.240 (0.740-2.079)	0.414
Hypertension	2.093 (1.287-3.403)	0.003	1.528 (0.943-2.478)	0.085
Diabetes mellitus	1.674 (0.771-3.634)	0.193	2.393 (1.114-5.142)	0.025
Hyperlipidemia	0.796 (0.452-1.403)	0.431	0.578 (0.329-1.016)	0.057
Previous vascular disease	1.507 (0.881-2.576)	0.134	2.205 (1.295-3.753)	0.004
Positive family history	1.800 (1.084-2.987)	0.023	1.753 (1.041-2.953)	0.035

eGFR indicates estimated glomerular filtration rate; BMI, body mass index; OR, odds ratio; and 95%, CI 95% confidence interval. All adjusted OR are adjusted for all other determinants listed.

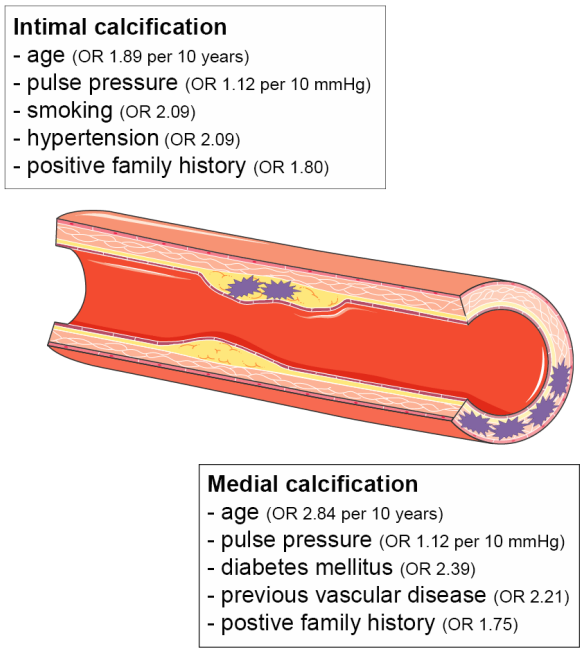

Figure 2 - Risk factors for intimal and medial arterial calcification
Risk factors for dominant intimal and dominant medial arterial calcification in multinomial regression. Figure prepared using templates from the Servier medical art Website (https://smart.servier.com).

Discussion

In this study we assessed the risk factors for the two types of arterial calcification, intimal and medial, that are known to affect the ICA, and determined whether a difference in risk profile exists between the dominant calcification types. Our study showed that patients with predominant medial calcification were older, more often female, smoked less often and more often had a history of previous vascular diseases, compared to patients with predominant intimal calcification. Multinomial regression confirmed the existence of differences in risk factors for predominant intimal and medial calcification. Older age, higher pulse pressure and positive family history were risk factors for both types of calcification. Whereas, smoking and hypertension were only risk factors for predominant intimal calcification, and diabetes mellitus and previous vascular disease were only risk factors for predominant medial calcification.

Although the differences are limited; the finding of differences in risk factors for the two calcification patterns support the concept that both types of calcification represent a difference in etiology.(17) Our findings are overall in agreement with previous studies in different vascular beds.(18) However, given the difficulty of separating medial and intimal calcifications in vivo, data on risk factors for the separate types of calcification are very limited. This literature is confined to some studies in breast arterial calcification (BAC) which is thought to be exclusively medial, and studies in which linear ^{18}F-sodium fluoride uptake in the femoral artery and high ankle brachial index are used as surrogate markers for medial arterial calcification.

For medial arterial calcification, previous studies also showed a relation between older age, diabetes mellitus, and previous vascular diseases.(18-21)

We did not find a relation between a previous diagnosis of hypertension and medial calcification. However, we did find an association with pulse pressure. The relation between medial calcification and hypertension has been investigated before, with conflicting results.(18,19,21,22) If a relation exists, one could speculate about the cause and effect. It could be that hypertension functions as a risk factor for atherosclerosis. However, the other way around medial calcification is thought to result in a decreased arterial compliance or stiffening of the arterial wall, which could lead to increased pulse pressure and hypertension.(23,24)

The relation between positive family history and medial arterial calcification differs from the sparse findings in literature, where no association was found.(21) The current found association between medial calcification and family history of cardiovascular diseases in this study could suggest that medial calcification

does play a role in the development of cardiovascular diseases. However, it could also mean that in case of cardiovascular diseases, due to vascular damage medial calcification develops more easily. A third hypothesis is that due to the presence of some shared risk factors, both intimal and medial calcification develop in the same families.

Different from previous studies, we did not find a direct or inverse relation between medial calcification and smoking. Although previous studies investigating the relationship between medial calcification and smoking have not been able to elucidate their interaction, a protective effect has been suggested in several of these studies.(12,19-22) As well, a recent study found an (unadjusted) inverse relation between overall, thus intimal and medial, ICAC severity and smoking (25) which may be explained by the compelling higher prevalence of medial calcification in the ICA (3) in combination with a possible protective effect of smoking on medial calcification.

For intimal calcification, the relation with smoking has extensively been described in literature, and was confirmed in our data.(26-28)

Furthermore, we found a clear relation between intimal calcification and hypertension. Hypertension can cause endothelial damage, resulting in impaired vascular contractility and proinflammatory activity, causing atherosclerosis.(29)

Previous literature does describe positive family history to be a risk factor for atherosclerosis (30) We could confirm this in our study.

Surprisingly, we did not find a relation between diabetes mellitus and intimal calcification, even though pathways of hyperglycemia leading to development, progression and instability of atherosclerotic lesions have been described in literature.(31) Also, we were not able to show a relation between previous vascular diseases or hyperlipidemia and intimal calcification.

This study has some important limitations, including the cross-sectional design and the inclusion of only patients with acute stroke or stroke-like symptoms. (Diagnosis at discharge: cerebral infarction in 89.3%, transient ischemic attack in 6.1% and another diagnosis in 4.6%.) The patients without calcifications were in general younger and suffered more often from acute stroke due to arterial dissection than the patients with calcifications. (Supplemental table V) It has been suggested that craniocervical artery dissection is triggered by a combination of both an underlying susceptibility (i.e. genetic, vascular anomaly, infection) and a (minor) mechanical trauma.(32) Furthermore, it is known that risk factors such as pregnancy, oral contraceptives and illicit drug use are related to ischaemic stroke in young adults (<50 year).(33) As we do not know if the differences in

risk factors for ischaemic stroke in the young population also propagate to a different cardiovascular risk profile as compared to healthy controls, we decided to exclude this population as a control population in this study. In the patients with indistinguishable calcifications, a comparable profile was seen, but less outspoken. Therefore, we chose to analyse the data in multiple ways and to compare the patients with predominant intimal and medial calcification with the patients with very small amounts of calcification (indistinguishable category). Another limitation of this study is the limited number of patients with decreased renal function, therefore we cannot draw firm conclusions on the effect of renal function. Furthermore, the used radiological scoring model in our study does not discriminate between intimal and medial calcification, but only distinguishes dominant calcification patterns. This means that patients with a dominant medial calcification pattern can, to variable extents, also suffer from intimal calcification, and vice versa. Nevertheless, this does not invalidate our results but might have diluted the associations.

In conclusion, we showed that the effect of risk factors on vascular calcification in the ICA depends on the location of these calcifications in the vascular wall, with age, pulse pressure, diabetes mellitus, previous vascular disease and positive family history being risk factors for medial arterial calcification and age, pulse pressure, smoking, hypertension and positive family history for intimal calcification. Our data support that these two types of calcifications represent different entities and support the hypothesis that a non-atherosclerotic pathway may also lead to stroke. This concept may evolve into novel strategies to prevent stroke in the future beyond atherosclerotic cardiovascular risk reduction.

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Supplemental table I - Characteristics in association with severity of intracranial carotid artery calcifications

	Absent n=119	Mild n=305	Moderate n=318	Severe n=390
Age (years)	48.6 ± 10.9	60.6 ± 12.1	70.2 ± 10.6	76.2 ± 9.1
Gender (% male)	66 (55.5%)	179 (58.7%)	188 (59.1%)	210 (53.8%)
Body mass index (kg/m^2)	26.3 (24.7-30.6)	26.4 (24.4-29.4)	26.6 (23.9-29.3)	25.7 (22.9-27.8)
Systolic blood pressure (mmHg)	146 (120-160)	150 (133-167)	158 (140-177)	159 (142-185)
Diastolic blood pressure	86 (75-100)	85 (75-91)	87 (78-98)	83 (73-94)
Pulse pressure (mmHg)	60.7 ± 16.6	67.7 ± 21.3	72.7 ± 22.0	78.4 ± 26.2
eGFR <60 mL/min/1.73m^2	5 (4.2%)	26 (8.6%)	35 (11.1%)	78 (20.2%)
Glucose (mmol/l)	6.4 (5.6-7.1)	6.5 (5.7-7.7)	6.7 (5.9-7.9)	6.8 (5.9-8.7)
Current smoker	40 (34.3%)	83 (28.8%)	91 (30.5%)	90 (25.7%)
Ever smoker	61 (52.1%)	192 (66.7%)	188 (63.1%)	209 (59.7%)
Hypertension	33 (28.0%)	117 (38.9%)	179 (56.5%)	266 (68.9%)
Diabetes mellitus	6 (5%)	29 (9.5%)	49 (15.4%)	87 (22.5%)
Hyperlipidemia	17 (14.4%)	96 (32.3%)	101 (33.1%)	174 (45.8%)
Previous vascular disease	28 (23.5%)	104 (34.1%)	146 (45.9%)	235 (60.3%)
Family history positive	37 (36.6%)	72 (28.9%)	70 (31.7%)	57 (25.7%)

Variables described as mean ± standard deviation for continuous variables, median (interquartile range) for skewed continuous variables, and number (%) for categorical variables

Supplemental table II - Association between risk factors and a predominant medial localized calcification pattern in patients with classifiable severe intracranial carotid artery calcifications*

Determinant	Crude OR (95% CI) for having predominant media calcification	P-value	Adjusted OR (95% CI) for having predominant media calcification	P-value
Gender (male)	0.343 (0.204-0.577)	<0.0005	0.382 (0.213-0.684)	0.001
Age (per 10 years)	2.210 (1.666-2.930)	<0.0005	1.681 (1.217-2.322)	0.002
BMI (kg/m^2)	0.968 (0.909-1.029)	0.294	0.971 (0.904-1.043)	0.416
Pulse pressure (per 10 mmHg)	1.057 (0.964-1.160)	0.236	1.019 (0.912-1.139)	0.738
eGFR <60 mL/min/1.73m^2	2.710 (1.294-5.675)	0.008	2.219 (1.000-4.924)	0.050
Current smoker	0.402 (0.239-0.677)	0.001	0.482 (0.256-0.908)	0.024
Hypertension	0.824 (0.489-1.390)	0.468	0.620 (0.336-1.146)	0.127
Diabetes mellitus	1.417 (0.775-2.590)	0.257	1.637 (0.822-3.259)	0.161
Hyperlipidemia	0.984 (0.611-1.585)	0.947	0.986 (0.538-1.806)	0.962
Previous vascular disease	1.512 (0.935-2.446)	0.092	2.044 (1.095-3.817)	0.025
Positive family history	0.765 (0.430-1.360)	0.359	0.942 (0.481-1.842)	0.860

eGFR indicates estimated glomerular filtration rate; BMI, body mass index; OR, odds ratio; and 95%, CI 95% confidence interval. All adjusted OR are adjusted for all other determinants listed.
*classifiable severe calcifications: all patients with a calcifications that could be scored as severe predominant intimal or severe predominant medial; patients without, with indistinghuisable, or with mild or moderate calcifications were not included in this analysis.

Supplemental table III - Association between risk factors and a predominant medial localized calcification pattern in patients with classifiable intracranial carotid artery calcifications (eGFR calculated as if all patients were black)*

Determinant	Crude OR (95% CI) for having predominant media calcification	P-value	Adjusted OR (95% CI) for having predominant media calcification	P-value
Gender (male)	0.549 (0.416-0.725)	<0.0005	0.662 (0.489-0.896)	0.008
Age (per 10 years)	1.680 (1.475-1.913)	<0.0005	1.522 (1.315-1.762)	<0.0005
BMI (kg/m^2)	0.974 (0.937-1.012)	0.183	0.980 (0.944-1.018)	0.291
Pulse pressure (per 10 mmHg)	1.058 (0.999-1.120)	0.054	1.010 (0.948-1.076)	0.760
eGFR <60 mL/min/1.73m^2	1.312 (0.770-2.236)	0.318	0.946 (0.534-1.676)	0.850
Current smoker	0.446 (0.326-0.609)	<0.0005	0.565 (0.400-0.797)	0.001
Hypertension	1.037 (0.787-1.363)	0.795	0.747 (0.539-1.037)	0.081
Diabetes mellitus	1.342 (0.933-1.930)	0.113	1.467 (0.978-2.200)	0.064
Hyperlipidemia	0.844 (0.638-1.117)	0.236	0.719 (0.504-1.025)	0.068
Previous vascular disease	1.302 (0.988-1.717)	0.061	1.493 (1.065-2.091)	0.020
Positive family history	0.765 (0.536-1.092)	0.140	0.985 (0.698-1.390)	0.932

eGFR indicates estimated glomerular filtration rate; BMI, body mass index; OR, odds ratio; and 95%, CI 95% confidence interval. All adjusted OR are adjusted for all other determinants listed.
*classifiable calcifications: all patients with calcifications that could be scored as predominant intimal or predominant medial; patients without calcifications or indistinghuisable calcifications were not included in this analysis.

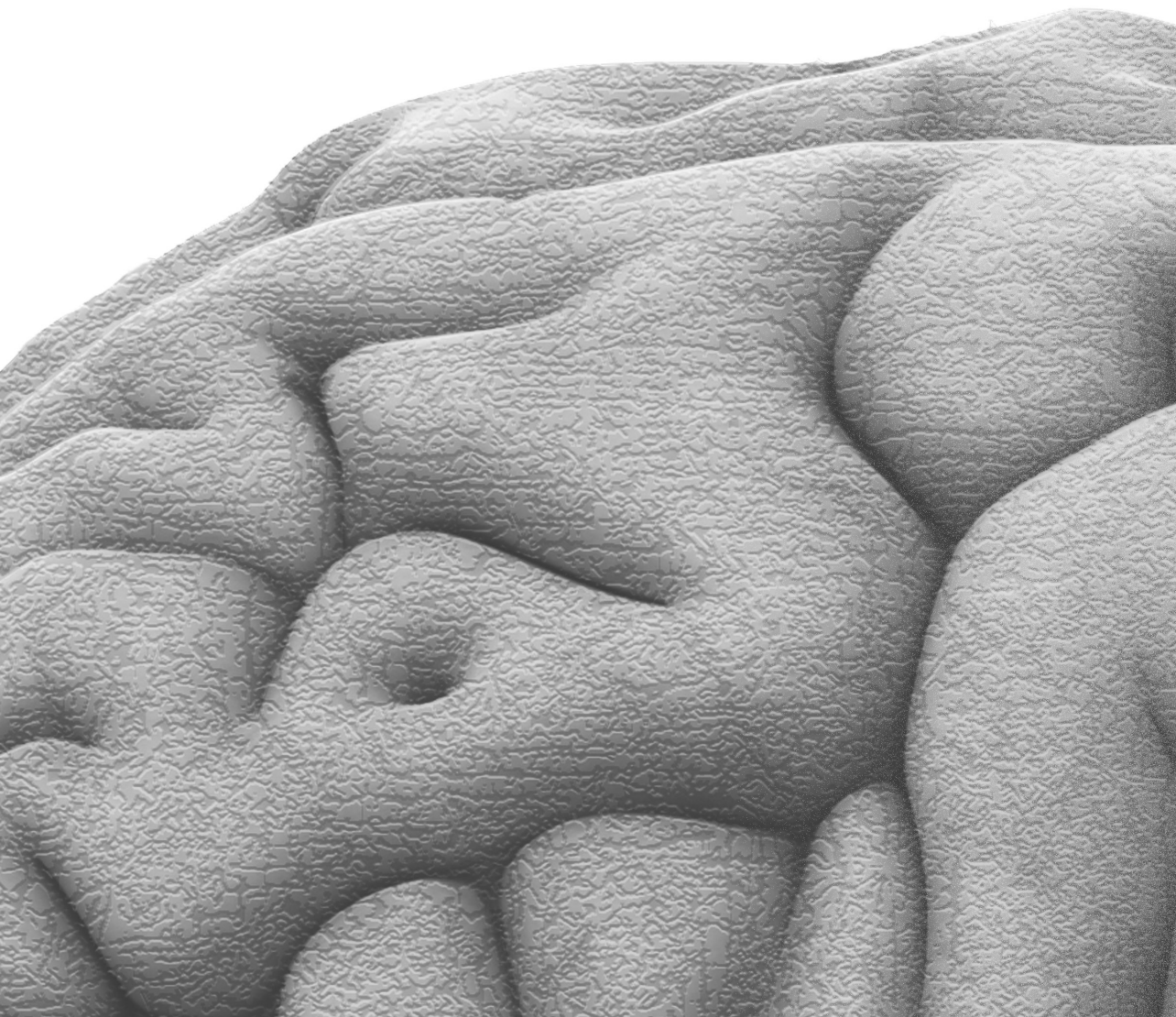
Supplemental table IV - Association between risk factors and a predominant intimal or predominant medial localized calcification pattern in the full cohort (eGFR calculated as if all patients were black)

Determinant	Adjusted OR (95% CI) for having predominant intima calcification	P-value	Adjusted OR (95% CI) for having predominant media calcification	P-value
Gender (male)	1.324 (0.849-2.065)	0.215	0.859 (0.553-1.334)	0.498
Age (per 10 years)	1.831 (1.492-2.247)	<0.0005	2.814 (2.280-3.474)	<0.0005
BMI (kg/m^2)	0.981 (0.933-1.031)	0.451	0.965 (0.917-1.014)	0.162
Pulse pressure (per 10 mmHg)	1.111 (1.004-1.240)	0.043	1.122 (1.011-1.246)	0.031
eGFR <60 mL/min/1.73m^2	0.793 (0.255-2.470)	0.689	0.746 (0.245-2.275)	0.607
Current smoker	2.091 (1.275-3.432)	0.004	1.236 (0.737-2.072)	0.421
Hypertension	2.034 (1.254-3.299)	0.004	1.508 (0.932-2.440)	0.095
Diabetes mellitus	1.648 (0.759-3.577)	0.206	2.387 (1.110-5.133)	0.026
Hyperlipidemia	0.803 (0.456-1.415)	0.448	0.580 (0.329-1.020)	0.059
Previous vascular disease	1.490 (0.872-2.546)	0.144	2.191 (1.287-3.728)	0.004
Positive family history	1.779 (1.074-2.948)	0.025	1.741 (1.035-2.928)	0.037

eGFR indicates estimated glomerular filtration rate; BMI, body mass index; OR, odds ratio; and 95%, CI 95% confidence interval. All adjusted OR are adjusted for all other determinants listed.

Supplemental table V - Cause of cerebral ischemia in patients with different types of intracranial carotid artery calcification

	Absent	Indistinguishable	Media	Intima
Large vessel disease	26 (33.3%)	32 (39.5%)	111 (46.3%)	172 (47.3%)
Cardiac emboli	16 (20.5%)	22 (27.2%)	59 (24.6%)	98 (26.9%)
Small vessel disease/ lacunar infarction	12 (15.4%)	16 (19.8%)	46 (19.2%)	69 (19.0%)
Dissection	19 (24.4%)	9 (11.1%)	7 (2.9%)	7 (1.9%)
Other	5 (6.4%)	2 (2.5%)	17 (7.1%)	18 (4.9%)



PART II

Hippocampal Calcification

CHAPTER 5

Histological validation of calcifications in the human hippocampus as seen on computed tomography

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Abstract

Background

Calcifications within the hippocampus were recently described for the first time on computed tomography (CT). These calcifications appeared in patients older than 50 years, the prevalence increases with age and they may be associated with cognitive decline. The aim of this study was to determine the histological basis (the presence, severity and location) of these CT-detected hippocampal calcifications of post-mortem brains.

Methods

CT scans of seven post-mortem brains were scored for the presence and severity (mild, moderate, severe) of hippocampal calcification. After this, samples from nine hippocampi (bilateral in two brains, unilateral in five brains) were stained with hematoxylin and eosin (HE) to indicate the cytoarchitecture, with Elastica van Gieson to analyse the elastic connective tissue of the vessel walls and with von Kossa for detection of calcium.

Results

In four brains (six hippocampi), calcifications were both found on CT and in corresponding histology. In three brains (three hippocampi), calcifications were absent on CT and corresponding histology. In histology, mild calcifications were located in the tail and severe calcifications involved the tail, body and sometimes the head of the hippocampus. The calcifications co-localised with precapillaries, capillaries and arteries of the molecular and granular layers of the dentate gyrus and the Cornu Ammonis 1.

Conclusions

In this study, calcifications of the hippocampus as seen on CT scans were histologically located in vascular structures of the tail, body and head of the hippocampus.

Introduction

Cerebrovascular disease is a major cause of morbidity and mortality worldwide. The main clinical diseases associated with intracranial vascular problems are stroke and dementia.(1) One of the phenomena commonly present in the intracranial vessels of cerebrovascular patients is the accumulation of calcium in the arterial wall. Large calcifications may cause occlusions and restrict blood flow to certain parts of the brain and calcifications may also stiffen arteries.(2,3) Calcifications of different brain structures can be demonstrated with computed tomography (CT). The most common calcifications of the intracranial carotid artery (4,3), the choroid plexus (5) and basal ganglia structures, like the globus pallidus and dentate nucleus (6, 7), have been observed on CT in human.

Calcification of the choroid plexus, which is responsible for the production of cerebrospinal fluid, increases in frequency at increasing age (5). The calcification can involve the temporal horn, the floor of the body of the lateral ventricle, the roof of the third ventricle and the foramen of Monro (5). These regions border the hippocampus, and calcifications of the hippocampus were therefore often misinterpreted as calcification of the choroid plexus.(8,9) The evolution of CT scan techniques allowed for better quality of images and thinner CT slices, which can be read more easily in various viewing planes. On these scans, a difference between choroid plexus calcifications and adjacent hippocampal calcifications can be made.

Limited research has been performed on calcifications of the hippocampus. Chew et al. (9) were the first to describe hippocampal calcification on CT. They found that hippocampal calcification appears particularly in patients older than 50 years and that the prevalence increases with age.(9)

A recent study by Kockelkoren et al. (8) investigated in a case-control study the presence of hippocampal calcification and the relationship with cognitive decline. In memory clinic patients, calcifications of the hippocampus were more prevalent and associated with a lower cognitive functioning.(8)

One histopathological study by Wegiel et al. (10) investigated calcifications located in the hippocampus in Alzheimer's disease, Down syndrome, and control aging patients. They described the calcifications to be a manifestation of vascular disease, which they called vascular fibrosis and calcifications.(10)

Currently, no evidence is available that the calcifications as observed on CT scans (8,9) are indeed corresponding to vascular fibrosis and calcifications as found in the histopathological study.(10) For this reason, the aim of this study was to determine the histological basis of these CT-detected hippocampal calcifications in human post-mortem brains.

Materials and methods

Patients

For this study, we examined a total of seven brains. One brain was derived from a body, which was included in a donation program from the Department of Anatomy. Six brains were from patients who had an autopsy at the Department of Pathology. Local ethical committee approval was obtained for research on retained tissues after written informed consent given by the patients during life or their next of kin after death (Medical Ethics Committee of the University Medical Center Utrecht 11-531/C). All brains were CT scanned post-mortem. **Table 1** shows the available information of the seven patients about age, gender and relevant medical history.

Table 1 - Patient information and relevant clinical history of the seven patients.

Patient number	Gender	Age (years)	Medical history	Number of hippocampi analysed
1	F	95	Body donation to the Department of Anatomy without available medical history	2 hippocampi: head, body and tail
2	F	87	Cerebrovascular Accident Atrial fibrillation	2 hippocampi: head, body and tail
3	F	62	Subarachnoid haemorrhage, intracranial aneurysm	1 hippocampus left
4	F	81	Atrial fibrillation Asthma Hypertension Sepsis and ischemia in leg	1 hippocampus right
5	M	60	Stem cell transplantation Human immunodeficiency virus Acute myeloid leukemia Sepsis and pulmonary infections Secondary inflammation of pituitary	1 hippocampus left
6	M	71	Sinus thrombosis Heart attack Polycythemia vera	1 hippocampus right
7	F	75	Haemorrhage in the thalamus and pons Atherosclerosis Atrial fibrillation Hypertension	1 hippocampus right

CT examinations

The brain CT scans were made with a Philips Brilliance 64-slice or 256-slice CT scanner (Philips Healthcare, Best, The Netherlands). The brain from the patient who donated her body to the Department of Anatomy was removed from the skull before scanning. The brains from the patients from the Department of Pathology were scanned surrounded by the skull. Non-contrasted thin slice reconstructions (0.8-1.0 mm) were analysed for hippocampal calcifications in different reconstructions, axial, coronal, and sagittal in the brain window setting (Center: 40 Hounsfield Units, Width: 80 Hounsfield Units) using the Philips IntelliSpace Portal 7.0 (Philips Healthcare, Best, The Netherlands). Calcifications were bilaterally scored on severity as absent, mild (one dot), moderate (multiple dots) or severe (confluent) as described by Kockelkoren et al..(8) Calcifications on the CT scans are seen as a group of white voxels with a density similar to bone (**Figure 2**). The hippocampi were scored by an experienced observer. His agreement in comparison with other observers was previously investigated (kappa 0.80). The observer was blinded to the histological results.

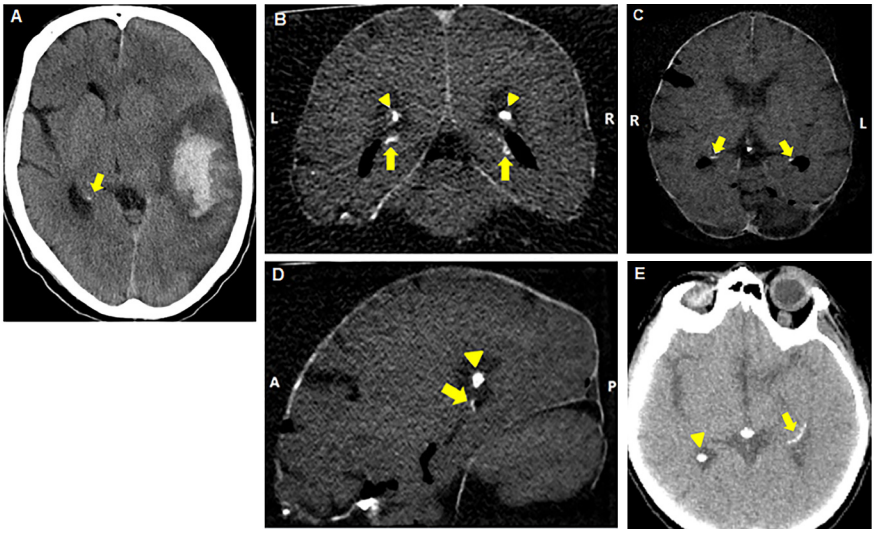

Figure 1 - Hippocampal calcifications on CT scans of patient numbers 4 (mild, A), 1 (moderate, B-D) and 3 (severe, E).
(A) Axial reconstructed image with mild hippocampal calcification (one dot). (B) Coronal reconstructed image shows (moderate) bilateral hippocampal calcification (multiple dots), indicated by arrows. Choroid plexus calcification is indicated by arrowheads. (C) Axial reconstructed image with moderate bilateral hippocampal calcification marked with arrows. (D) Sagittal reconstructed image with moderate hippocampal calcification marked with an arrow and choroid plexus calcification marked with an arrowhead. (E) Axial reconstructed image shows severe hippocampal calcification (confluent) indicated by an arrow and calcification of the choroid plexus is indicated with an arrowhead.

Microscopy – Histological study of nine hippocampi of seven patients

In two patients (patient number 1 and 2, corresponding with **table 1 and 2**), both hippocampi (hippocampus number 1 to 4, corresponding with **table 2**) were evaluated by histology, in five patients (patient 3 to 7, corresponding with **table 2**) one hippocampus was evaluated. The tail, body and head of the hippocampus were sampled in four of the nine hippocampi (hippocampus number 1 to 4). In the other five hippocampi, only the body was evaluated (due to the standard procedure of the Department of Pathology, in which the diagnosis of the disease the patient suffered from is the most important purpose. For each patient 22 standard pieces of the brain were cut out and three pieces with own content, these three extra pieces for diagnosis of the disease were more important than pieces for research purposes. This is why not always the head, body and tail of the hippocampus were analysed). Samples of the brains were put into cassettes. All cassettes were dehydrated in a graded series of ethanol (up to 95%) and embedded in paraffin. Subsequently, the paraffin embedded samples were cut into slices of 6 μm and put on glass microscope slides for pathological study. All sections were stained with hematoxylin and eosin (HE) for characterization of the cytoarchitecture.(10) In addition to this, the hippocampal sections were stained with von Kossa method for detection of calcium (10) and Elastica van Gieson stain to identify elastic connective tissue in vessel walls.(11) An experienced neuropathologist analysed all the specimens. The pathologist was blinded to the results of the CT scan. The calcifications in histology were quantified by the amount of vessels that were positive for calcium. Less than five vessels affected with calcium was considered mild, more than five non-confluent calcified vessels affected was considered moderate and more than five confluent calcified vessels that often had large calcium beads was considered severe.

Macroscopy of the brain of one patient

In one patient (patient number 1, as described in **table 1 and 2**), a highly detailed analysis of the whole brain and both hippocampi was performed, to find more detailed information about the location of the calcifications in the vessel structures. This particular brain was obtained from the Department of Anatomy and had been fixed in 3% buffered formaldehyde. The brain was cut into coronal slices of 10 mm thickness, 25 samples were taken and put into cassettes. **Figure 2** shows a coronal slice of the posterior part of the brain. The hippocampus as seen in **figure 2B** was removed into a cassette and used for histological study. The important samples are bilateral hippocampus anterior (head), middle (body), and posterior (tail), beside the other samples for general microscopy.

Results

Computed tomography

Calcification of the hippocampus was found bilaterally in six of the nine hippocampi on CT scan. Three hippocampi had severe calcifications, two had moderate calcifications, one had only mild calcification and three had no calcifications. **Figure 1** shows, as example, the routine CT images in different planes with hippocampal calcification.

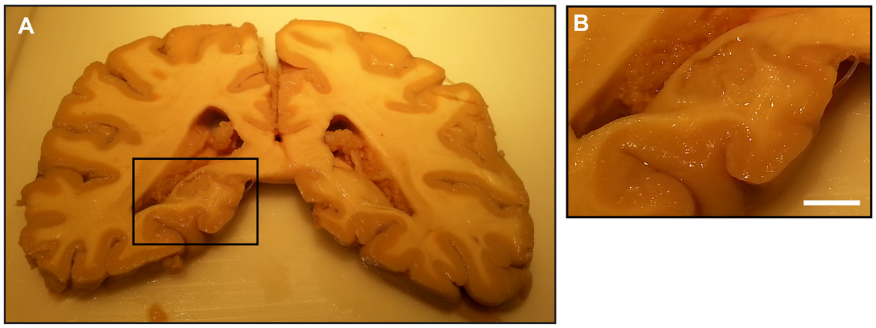

Figure 2 - Coronal brain slice after dissection of the brain (of patient number 1).
(A) Coronal slice with the posterior part of the hippocampus (tail). (B) Close up of the posterior part of the hippocampus (scale bar = 10 mm). Choroid plexus is visible in the lateral ventricle. The close up shows the sample for further histological staining.

Correlation of CT findings with histology

In all six hippocampi with calcifications and all three hippocampi without calcifications on CT, these results from CT were confirmed by histology. Of the six calcified hippocampi three were considered as severe calcifications, two as moderate and one as mild. The correlation between calcifications on CT and the histopathological findings is shown in **table 2**. Validated with histological staining, four subjects had severe calcifications, two had mild calcifications, and three subjects had no calcifications.

Vascular localization of hippocampal calcification

We analysed one brain with two hippocampi (patient number 1, as described in **table 1 and 2**) in more detail than the other specimens. To indicate the exact location of the calcifications in the vessel structures.

In the anterior part, the head of the hippocampus, no calcification was noticed, neither in the left nor in the right hippocampus. The middle part, the body of

Table 2 - Overview of hippocampal calcifications on CT scan and in histological study.

	CT scan Severity	Histology Severity
Hippocampus number		
1	++	+++
2	++	+
3	+++	+++
4	+++	+++
5	+++	+++
6	+	+
7	-	-
8	-	-
9	-	-

Severity: whether the calcification is severe +++, moderate ++, mild + or absent -.
CT scoring: absent, mild (one dot), moderate (multiple dots) or severe (confluent) (8).
Histology scoring: absent, mild (less than five vessels affected with calcium), moderate (more than five non-confluent calcified vessels) or severe (more than five confluent calcified vessels that often had large calcium beads).

the left hippocampus, showed mild calcification localized in the precapillaries and capillaries in the molecular layer of the dentate gyrus (DG) and Cornu Ammonis 1 (CA1).

The posterior part, the tail of the left hippocampus, showed severe calcification of precapillaries, capillaries and the arteries (**Figure 3**). These calcification beads, shown in **Figure 3C**, were larger in comparison to the mild calcification observed in the left hippocampal body. Also, an increase of number of calcifications was noticed. In this severe stage, calcifications were localized in the granular layer of the DG and the molecular layer of the DG and CA1 and spread out over the border of CA1 into the molecular layer of the subiculum (**Figure 3B**). **Figure 3D** shows a calcified artery in the molecular layer of the CA1. The Elastica van Gieson stain clearly showed the structure of an artery, with calcification in the tunica adventitia and the tunica media, as seen in **figure 3E**. In some slices, it was difficult to distinguish the structure of the vessels in this severe stage of calcification, because sometimes no vessel walls were observed, only the calcium deposits.

The right hippocampus contained no calcification in the body. The posterior part, the tail of the right hippocampus, showed mild calcification in precapillaries and capillaries (**Figure 4**).

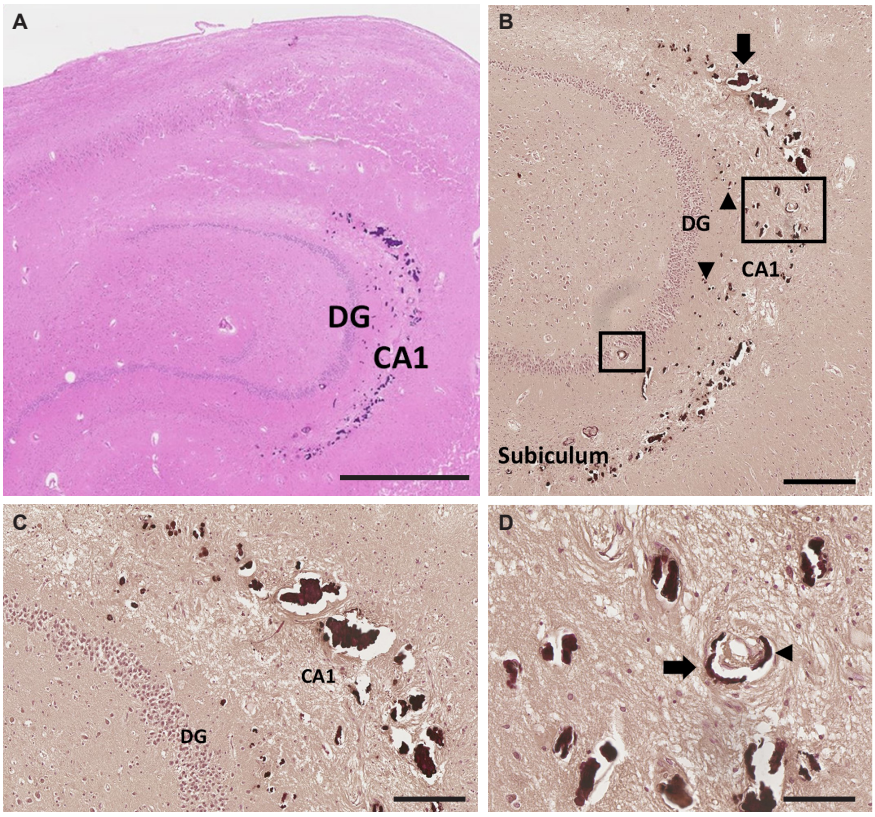

Figure 3 - Severe calcifications in the tail of the left hippocampus (hippocampus number 1) of patient number 1 (as described in **table 1 and 2**).
(A) HE stain overview of the left hippocampal tail. Scale bar = 1 mm, 4x magnification. (B) Von Kossa-positive deposits in precapillaries and capillaries (arrowheads) in the molecular layer of the DG and CA1. Calcifications of the bigger vessels, mostly arteria (arrow), in the molecular layer of CA1 are clearly observed. Two arteria which are calcified are surrounded, one in the molecular layer of the CA1 and one in the granular layer of the DG. The calcifications spread out into the molecular layer of the subiculum. Scale bar = 600 μm, 10x magnification. (C) Zoomed in on the big calcifications of precapillaries, capillaries and bigger vessels in the molecular layer of the DG and CA1. Epithelial cells of the vascular wall are not seen, because of the big calcification deposits. Scale bar = 400 μm, 20x magnification. (D) Enlarged image of a von Kossa-positive calcified artery in the molecular layer of the CA1. Calcification of the tunica adventitia (arrow) and the tunica media (arrowhead) are identified. Scale bar = 200 μm, 40x magnification. (E) Calcified artery in the granular layer of the DG shown with Elastica van Gieson stain. Calcification of the tunica adventitia (arrow) and the tunica media (arrowhead) are identified. Scale bar = 100 μm, 40x magnification.

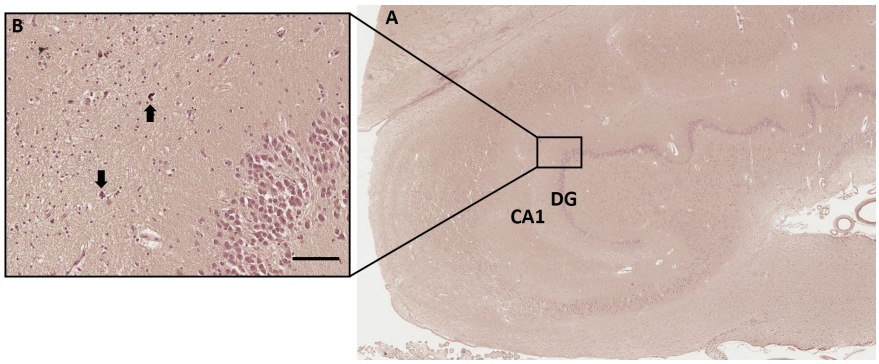

Figure 4 - Calcification of precapillaries and capillaries in the right hippocampal tail (hippocampus number 2) of patient number 1 (as described in **table 1 and 2**).
(A) Overview of the right hippocampal tail. Scale bar = 1 mm, 4x magnification. (B) Zoomed picture of von Kossa-positive stain, calcifications are located in the precapillaries and capillaries (arrows) in the right hippocampal tail. Scale bar = 100 μm, 40x magnification.

Onset of calcification

In the two brains (patient number 1 and 2, as described in **table 1 and 2**) of which the tail, body and head of both hippocampi were analysed, a striking pattern was seen. In one brain, both hippocampi were severely calcified in the tail, moderately calcified in the body and mildly calcified in the head. In the other brain, the tail of one hippocampus was affected by severe calcification and the body of that hippocampus showed mild calcification. In the other hippocampus only the tail was mildly calcified. This calcification pattern indicates that the calcification first appears in the tail, followed by the body, and subsequently when severe also occurs in the head of the hippocampus. This remains speculative and needs further confirmation.

Choroid plexus calcification

To demonstrate the difference between hippocampal calcification and calcification of the choroid plexus, a picture of choroid plexus calcification is included (**Figure 5**). Small vessels, precapillaries and capillaries were calcified in the choroid plexus. The large arteries and veins were less calcified.

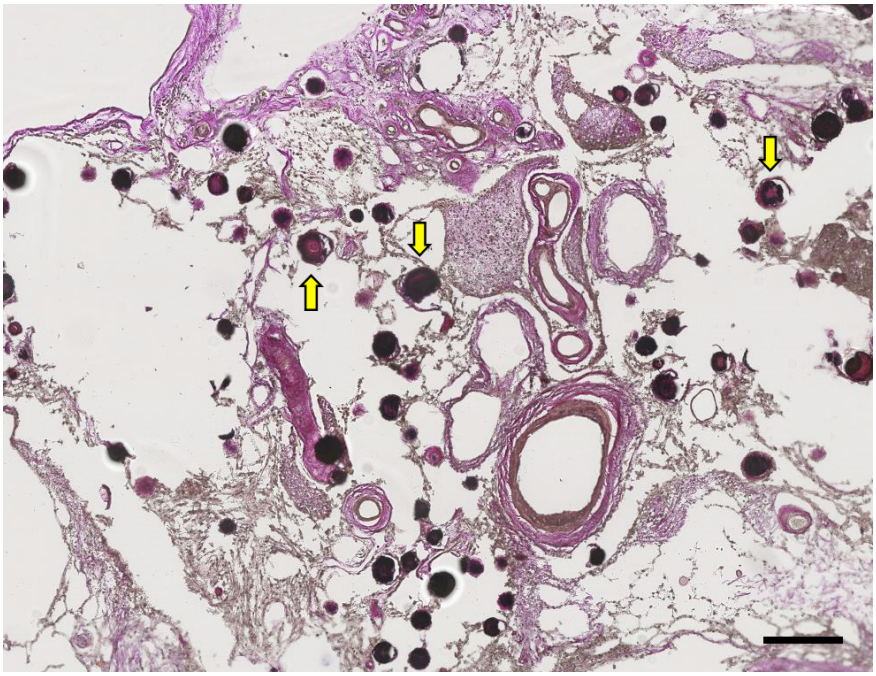

Figure 5 - Calcification of the choroid plexus on the left side. The dark red/black dots, some are indicated with yellow arrows, are calcifications of precapillaries and capillaries in the choroid plexus on the left side of the brain.

Discussion

The main finding of this study is that hippocampal calcifications as observed on CT images are confirmed with histology to be located in the hippocampal vasculature. Mild calcifications were found posterior in the tail of the hippocampus and extended into the body, toward the head of the hippocampus in severe stages. Whether this is a pattern that repeatedly will occur in different patients, should be further investigated and confirmed. The calcifications started in the precapillaries and capillaries of the molecular layer of the DG and CA1. Severe calcification expands in the molecular layer of the DG and CA1, the granular layer of the DG and over the border of CA1 to the molecular layer of the subiculum, as observed in four severe calcified hippocampi. The vascular calcifications seemed to be located in the adventitial and medial layer of the vessel wall. Our study is the first study that confirmed the vascular origin of the hippocampal calcifications that are detectable on CT.

Limited research has been done on calcifications of the hippocampus. A histopathological study from 2002 described vascular fibrosis and calcification in the hippocampus in subjects with Alzheimer's disease (in 59% of the patients), Down syndrome patients (in 4%) and control aging patients (in 40%).(10) In that study, just as in ours, calcifications were found in precapillaries and capillaries of the molecular layer of the DG and expanded to the granular layer and polymorphic layer of the DG, and to the molecular layer of the CA1. These calcifications found in the study of Wegiel et al. (10) apparently start in the hippocampal tail and spread in severe stages to the body and in some cases to the head of the hippocampus. A finding that we have seen as well in two brains of which the tail, body and head of the four hippocampi were analysed. This pattern remains speculative and needs further confirmation. In the study of Wegiel et al. (10) was found that the calcifications caused changes of the endothelial cells in the wall and lumen of vessels. In later stadia, the vascular wall became thicker and the calcified beads increased, these calcium deposits degraded the vessels and occlusion of blood circulation was noticed. Which indicates that an early diagnosis of these calcifications, with for example CT, will be valuable. Also in our study we observed degraded vessel walls and big calcium deposits, which might be implicated in the pathophysiology of vascular degradation. Our study demonstrated, through comparison with histological staining, that the calcifications found on CT images are indeed located in the hippocampal vasculature. The hippocampal calcifications are located in the molecular layer of the DG and CA1 and in the granular layer of the DG as observed in all analysed hippocampi positive for hippocampal calcifications. Interestingly, these locations correspond with the location of vascular fibrosis and calcifications described in the histopathological study of Wegiel et al..(10)

Calcifications can be detected by neuroimaging, and are seen as high density areas on CT scans, while these calcification areas are considerable less visible on magnetic resonance imaging (MRI).(12) Indicating that CT will provide a better diagnosis for hippocampal calcifications than MRI. A second study from 2012 (9) was the first study to describe hippocampal calcifications on CT images. Due to thin slice CT images, a distinction between choroid plexus calcifications and hippocampal calcifications could be made. In total, 300 randomly selected CT scans were analysed, of which intrahippocampal calcification was demonstrated in 47 patients, all these patients were older than 50 years of age. The authors concluded that intrahippocampal calcification appeared with increasing age.(9) With our study we demonstrated with histology that the CT detected calcifications were indeed located in the hippocampus.

The effect of hippocampal calcifications on cognitive functioning was investigated in a recent pilot study from 2016.(8) Kockelkoren et al. (8) examined memory clinic patients and controls. In this study, it was found that hippocampal calcifications were three times as prevalent in patients of the memory clinic compared to control patients. Furthermore, memory clinic patients with hippocampal calcifications showed lower cognitive functioning measured with Mini Mental State Exam. In two other smaller studies no significant difference in the presence of hippocampal calcifications between controls and a group of Alzheimer's disease patients was found.(10,13) More research should be performed to possibly confirm the correlation of hippocampal calcifications and dementia. In this study of Kockelkoren and colleagues it was hypothesised that the hippocampal calcification as seen on CT scan could be caused by vascular fibrosis and calcification, because it seems like it is located in the same region of the hippocampus.(8) Until now, this remained speculative, but our study provides support for this hypothesis by correlating CT findings to histology in the same brain.

In the Alzheimer's disease patients in the study of Wegiel et al. (10), neuronal cell loss was found in the CA1 region and subiculum proper, comparable to hippocampal sclerosis.(10) This was suggested to be characteristic for Alzheimer's disease patients. In normal aging patients, neuronal cell loss was rarely seen in the CA1 region of the hippocampus.(12) In our study we used material of normal aging patients. In histology we observed that the patients had some signs of aging, like amyloid-beta senile plaques, which was age-appropriate. We did not find obvious neuronal cell loss, which may be due to the fact that our subjects did not have Alzheimer's disease.(14)

The posterior cerebral artery supplies the hippocampus of blood. According to the flow territories, the branches of the hippocampal arteries can be divided into two groups. Blood supply to the body and tail of the hippocampus is from the middle and the posterior hippocampal arteries. The anterior hippocampal arteries supply the head of the hippocampus and the uncus.(15) Also the choroid plexus of the lateral ventricle is supplied by branches of the posterior cerebral artery.(16) Since the calcification in the vasculature in both the hippocampus and the choroid plexus is similarly located in the precapillary and capillary vessels, and both types of calcification have the same blood supply from the posterior cerebral artery, future research could be directed to a possible relation between these calcifications.

Future research may also focus on the effects of hippocampal calcifications on cognitive functioning. The main function of the hippocampus is learning and memory. Nowadays it is thought that the hippocampal sub regions, anterior

(head) and posterior (tail), are involved in different aspects of memory. The exact distribution is still not known and different studies suggest different functions.(17,18) Because the hippocampal tail is affected most early in this calcinosis process, it would be interesting to investigate the function of the posterior hippocampus in relationship with hippocampal calcifications. A potential link with cognitive decline and dementia could be investigated not only in memory clinic patients, but also in normal aging patients. In the limited medical history of our patients, we found that some patients had neurological damage and others had cardiovascular problems, both of which may be associated with calcifications in the brain. In future research a possible association between cardiovascular diseases and hippocampal calcifications can be investigated.

This study, in which we validated hippocampal calcifications as observed on CT scan with histological staining, is a crucial step for future research to investigate the underlying mechanism and consequences of these hippocampal calcifications. In the future we could detect the hippocampal vascular calcifications in early stages with thin slice CT scans. Thin slice CT scans could become a prognostic marker for cognitive decline (8) or other not yet investigated consequences of hippocampal calcifications. When finally the mechanism underlying hippocampal calcifications is known and this is related to a negative function for the patients, treatments on this mechanism could be investigated. With the prognostic marker of CT scans and applicable treatments, patients can be helped earlier.

Limitations of the current study are the lack of information about cognitive ability of the patients. Nothing can be said about a possible correlation between hippocampal calcifications and cognitive functioning of these patients. Secondly, the sample size of this study was relatively small, however, it is in our opinion sufficient to validate the calcifications on CT scans with histology, the main focus of this research. In the histology of four hippocampi we saw a possible pattern of calcification severity that expanded from tail toward the head of the hippocampus, although this is a limited number, the pattern was also observed by Wegiel et al..(10) We think further studies with larger cohorts who underwent CT scanning can more firmly investigate this pattern and we did not specifically investigate this in the present study. A last limitation is the way we quantified the histological findings. There is not yet a clear standard for the quantification of hippocampal calcification in histology as it is the case for hippocampal calcifications on CT scan.(8) Therefore, we made an own quantification method for hippocampal calcification for histology.

In conclusion, we showed that all hippocampal calcifications observed on thin slice CT images are confirmed by histological staining to be of vascular origin. The calcifications in mild stage were found in precapillaries and capillaries of the molecular layer of the DG and CA1. In a more severe stage also the arteries in the molecular layer of the CA1 and the granular layer of the DG were affected. Calcifications were most often found in the hippocampal tail and in a more severe stage they also became visible in the hippocampal body and sometimes in the hippocampal head. In further studies, the consequences of these hippocampal calcifications on cognitive impairment or possible other functions of the hippocampus, can be investigated.

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CHAPTER 6

Hippocampal calcification on computed tomography in relation to cognitive decline in memory clinic patients: a case-control study

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Abstract

Background

It was recently shown that calcification of the hippocampus can be detected on computed tomography (CT) images and these calcifications occur in up to 20% of people over 50 years of age. However, little is known about hippocampal calcification and its relation to cognition and cognitive decline. Therefore, the aim of this study was to; (1) determine the prevalence of hippocampal calcification on CT in memory clinic patients and controls, and (2) to assess its relation with cognitive decline.

Methods

67 patients from a memory clinic (cases) were matched by age and gender to a control group. In both groups, hippocampal calcification was assessed by two raters on thin slice, non-contrast enhanced brain CT images. Calcifications were scored bilaterally on presence and severity (absent, mild, moderate, severe). Mini Mental State Exam (MMSE) score was determined in cases.

Results

Hippocampal calcification presence was significantly higher in cases (N=26, 38.8%) compared to controls (N=9, 13.4%) (P<.01) with an odds ratio of 4.40 (95%CI: 1.63-14.87). In cases, MMSE score was significantly lower in those with hippocampal calcification compared to those without (21.6 vs 24.5, p=.02).

Conclusion

In this case-control study we found significantly more hippocampal calcification in patients with cognitive decline as compared to controls. Furthermore, within the cases, MMSE score was significantly lower in those with hippocampal calcification.

Introduction

Calcification of the hippocampus as seen on computed tomography (CT) is a relatively unknown radiological finding, frequently overlooked or misinterpreted as calcification of the adjacent choroid plexus.(1) Due to the increased image quality of CT scanners and more common use of thin slice imaging and multiplanar reformatting techniques, choroid plexus calcification and hippocampal calcification (HCC) can now be viewed independently. Applying these improved CT techniques, the first radiological study on this subject showed that HCC is a relatively frequent finding in the elderly occurring in up to 20% of subjects over 50 years of age.(1)

HCC has previously been described in histopathological literature where it was postulated that HCC is related to Vascular Fibrosis and Calcification (VFC) of the hippocampus, a form of vascular pathology which results in neural loss in affected subjects.(2) Despite this finding no further studies investigated HCC and its possible relation to cognition and cognitive decline. One important factor which could contribute to this is the fact that magnetic resonance imaging (MRI), the main imaging modality in neurocognitive research, is considerably limited in visualizing calcifications.(3)

The new insights gained in the detection of HCC with CT, the high prevalence of HCC, and the knowledge of VFC being related to neural loss have led us to investigate the relation between HCC and cognitive decline. In this pilot case-control study the prevalence of HCC in patients referred to a memory clinic for cognitive problems was compared with the prevalence of HCC in control subjects, and the relation of HCC with cognitive function was studied.

Methods

Patients

Our institutional review board approved this study (approval number: 15/432). Informed consent was waived due to the retrospective nature of the study. Inclusion criteria for the cases were; (1) patients referred to our memory clinic between 2009 and 2015 because of cognitive complaints and (2) a non-contrast enhanced brain CT examination within one year of the visit to the memory clinic. In the subjects referred to the memory clinic, global cognitive function was assessed by the treating physician using the Mini Mental State Examination (MMSE).(4)

The control group consisted of high energy trauma (HET) patients who were examined in our center between 2011 and 2015. Trauma patients were chosen over other patient groups as they represent a random sample of the general population due to the random nature of traumas. In our center, all HET patients undergo a total body CT scan to exclude intracranial pathology. Patients with extensive

intracranial hemorrhage on CT were excluded from the study. The controls were matched by gender and age to the cases with a maximum age-difference at the time of the CT of one year.

CT examinations

Brain CT examinations were acquired on a Philips Brilliance 64-slice or 256-slice CT scanner (Philips Healthcare, Best, The Netherlands). Patients were scanned from the skull base to the vertex. The non-contrast enhanced thin slice reconstructions (0.625 – 1 mm) were rated blinded and individually by two experienced radiologists with 14 and 16 years of experience in reading (brain) CT scans. Images were analyzed in axial, coronal and sagittal plane in the brain window setting (Center: 40, Width: 100) using the Philips IntelliSpace Portal 7.0 (Philips Healthcare, Best, The Netherlands). Calcifications were scored bilaterally in the hippocampus as absent, mild (one dot), moderate (multiple dots) or severe (confluent) (**Figure 1**). Calcifications are seen in the brain CT window setting as white (clustered) dense configurations comparable to bone and had to be clearly located in the hippocampus. In case of disagreement between both readers, a consensus reading determined the final score.

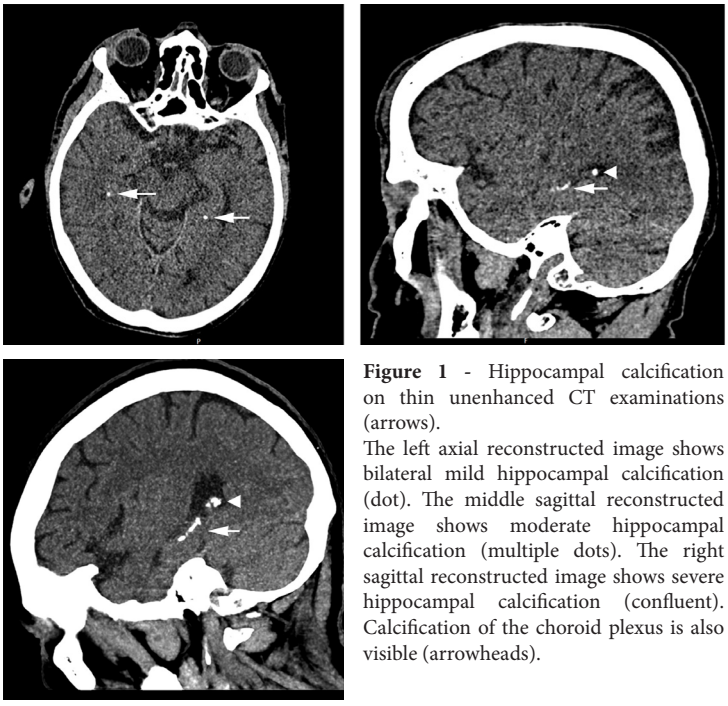

Figure 1 - Hippocampal calcification on thin unenhanced CT examinations (arrows).
The left axial reconstructed image shows bilateral mild hippocampal calcification (dot). The middle sagittal reconstructed image shows moderate hippocampal calcification (multiple dots). The right sagittal reconstructed image shows severe hippocampal calcification (confluent). Calcification of the choroid plexus is also visible (arrowheads).

Statistical analyses

Descriptive statistics, means/medians and 95% confidence intervals for continuous variables and counts and percentages for categorical variables were applied to describe the characteristics of the cohort. Spearman's rank correlation coefficient was used for correlation analyses. McNemar's test for paired nominal data was used to compare HCC presence in cases and controls and Students t-tests were used for determining differences in continuous variables. Interobserver agreement of HCC presence (yes/no) was calculated using kappa statistics.(5) A p-value of <0.05 was considered significant. Statistical analysis was performed using SPSS (IBM SPSS Statistics, Version 23.0. IBM Corp, Armonk, NY).

Results

The final cohort consisted of 67 cases, 32 men and 35 women and an equal number in the control group. The median number of days between the memory clinic visit and brain CT examination was 60 (range 0 to 329 days). Cases and controls were 77.2 (range 49-95 years) and 76.8 years of age (range 49-95 years) respectively. The mean age of women (79.1 years) was significantly higher than that of men (74.7 years) (P=.033). HCC was observed more often in cases (N=26 [38.8%]) compared to controls (N=9, [13.4%]), with an odds ratio of 4.40 (95%CI: 1.63-14.87, p<0.01) (**Table 1**).

Table 1 - Hippocampal calcifications in cases and controls by age and gender

	Memory Clinic Group		Control Group	
	Patients	**HCC**	**Patients**	**HCC**
Age (years)				
<70	14 (21)	2 (14)	14 (21)	3 (21)
70 – 80	26 (39)	12 (46)	26 (39)	1 (4)
>80	27 (40)	12 (44)	27 (40)	5 (19)
Total	67 (100)	26 (39)	67 (100)	9 (13)
Gender				
Male	32 (48)	15 (47)	32 (48)	3 (9)
Female	35 (52)	11 (31)	35 (52)	6 (17)

HCC = Hippocampal Calcification. Values are presented as n (%)

In the cases the distribution of severity of HCC was 13 mild, 6 moderate and 7 severe and in the controls 3, 1 and 5, respectively (**Table 2**). Mild calcifications were always found in the hippocampal tail while moderate and severe calcifications extended further towards the hippocampal head. The calcifications were located in the lateral segment of the hippocampus adjacent to the temporal horn of the lateral ventricles. There was no significant difference in the prevalence of HCC between men and women in the cases and controls being 46,9% and 31,4% (p=.195) and 9.3% and 17.1% (p=.352), respectively.

Table 2 - Hippocampal calcifications severity and laterality in cases and controls

	Memory Clinic Group	Control Group
HCC Severity		
Absent	41 (61)	58 (87)
Mild	13 (19)	4 (6)
Moderate	6 (9)	3 (4)
Severe	7 (10)	2 (3)
HCC Side		
Left	2 (8)	1 (11)
Right	9 (35)	3 (33)
Bilateral	15 (57)	5 (56)

HCC = Hippocampal Calcification. Values are presented as n (%)

In cases and controls combined, HCC severity increased with age (r=.20, P=.02). Patients with HCC were on average older (79.6 years) as compared to patients without HCC (76.1 years) (P=.04). Calcifications occurred most frequently bilaterally (N=20 [57.1%]), followed by the right hippocampus (N=12 [34.3%]) and left (N=3 [8.6%]).

MMSE was available in 56 cases and was lower in patients with HCC (21.6 points [95%CI 19.3-23.9]) compared to those without (24.5 points [95%CI 23.1-25.9], p=.02). Interobserver agreement of HCC presence was good with a kappa of .80.

Discussion

In this pilot case-control study we showed that HCC as seen on CT images is three times more common in patients visiting a memory clinic for cognitive problems compared to control patients, and that cognitive functioning in these patients, as measured by MMSE, is worse when HCC is present.

Calcification of the hippocampus is still relatively unknown and has only been examined in two studies, one from 2002 focusing on histopathology(2) and one from 2012 on CT imaging.(1) In the histopathological study it was shown that hippocampal VFC occurred in up to 60% of patients with Alzheimer's disease and 40% of a control group. In this study, it was found that VFC always started in the tail of the hippocampus and only progressed in a limited number of cases to the body and head. The calcifications were located in the wall of precapillary and capillary vessels within the flow territory of the middle hippocampal artery and were described as 'idiopathic non-atheriosclerotic'. In the more severe cases VFC led to loss of vessel wall structure and disappearance of nearly all neurons in the dentate gyrus, CA1 sector and subiculum proper.(2) The authors concluded that VFC in the hippocampus may contribute to or cause hippocampal sclerosis, a disease that has been linked to cerebrovascular pathology and dementia in the elderly.(6,7)

Thus far only one CT imaging study looked into HCC and concluded that it is a common finding in patients older than 50 years, contradicting previous radiological literature which suggested that HCC only occurred in rare conditions.(1) They further concluded that thinslice, multiplanar CT allows for adequate distinction between choroid plexus calcification and HCC, which was confirmed by the good interrater agreement in our study (kappa: .80).(1) Unfortunately, within their study, analysis of the effect of calcification on cognitive functioning was hindered due to small sample size and absence of cognitive testing. Interestingly, calcifications as visualized by CT were also found to always start in the tail of the hippocampus and in some cases progress to the body and head. Thereby, it was hypothesized that the HCC seen on CT scan could be caused by VFC. In our study HCC, and VFC also appeared to be located in the same segment within the hippocampus. HCC was found throughout the lateral segment of the hippocampus which correlates to the dentate gyrus and CA1 sector, the area where the calcified vasculature was described by Wegiel et al in their histological study.(2) If HCC and VFC, two calcifying processes that originate in the hippocampal tail, were to be related it could also explain the apparent relation we found in our study between HCC and cognitive decline. Recent studies investigating the functions of the hippocampus along its longitudinal axis concluded that the tail is most sensitive to ischemia and that the dentate gyrus is the most likely place where age related cognitive decline could start.(8–10) Possibly the calcifications as seen on CT are related to VFC and reflect these aging related changes. VFC was also found in patients with Alzheimer's disease. However, Alzheimer's disease is thought to originate in the enthorhinal cortex which is located around the head of the hippocampus. Thus,

based on the location in the hippocampus, we believe that the calcifications as visualized in our study, mainly localized in the tail, are more related to aging related cognitive decline. However, more in-depth studies are required to ensure that the calcification patterns in cognitive decline are distinct from the calcification pattern in Alzheimer's disease.

CT and MRI are currently the only radiological modalities that are capable of visualizing calcifications in the brain. MRI is the most frequently utilized imaging modality in neurocognitive studies because of the high spatial resolution, soft tissue contrast, functional imaging capabilities and lack of ionizing radiation. The literature on imaging of intracranial calcifications using MRI is sparse because of inherent limitations of the modality.(3) Calcifications on MRI have various signal intensities on conventional T_1- and T_2-weighted acquisitions which makes definitive identification of calcifications difficult.(11,12) The best MRI acquisition technique for the detection of calcification would be susceptibility weighted imaging (SWI), however, this sequence is not part of a standard 'cognitive imaging package' and signal intensities of calcification overlap with those of other signal sources (e.g. blood). The accuracy of SWI in the detection of hippocampal calcification has not been assessed so far.

CT scans are used far less frequently in neurocognitive research. CT is based on x-ray attenuation of tissues and excels in visualizing high contrast areas. Because there is little variation in the density of brain tissue CT scans cannot pick up on subtle differences which MRI can. On the other hand, dense and high contrast tissues like calcifications are easily distinguished. While MRI will remain the primary modality for neurocognitive studies, with the increasing evidence on prognostic CT markers for cognitive decline and stroke and the dramatic decrease in radiation dose, CT could see an increase in applicability in this field.(13–15)

The presented study is limited by the fact that the cognitive status of the control group was unknown. This could have led to underestimation of the difference in prevalence between cases and controls. Secondly the sample size of the study was relatively small which precluded multivariable analysis. However, the difference in presence of HCC between cases and control was substantial which supports the validity of our results.

In conclusion, we found a significantly higher prevalence of HCC on CT in patients referred to a memory clinic for cognitive problems compared to matched controls and HCC was associated with worse cognitive functioning. Larger studies will be required to further analyze and determine the role of HCC in cognitive decline and its relation to vascular disease.

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CHAPTER 7

Hippocampal calcification on brain CT: prevalence and risk factors in a cerebrovascular cohort

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Abstract

Background

Recently, hippocampal calcification as observed on brain CT examinations was identified in over 20% of people over 50 years of age and a relation between hippocampal calcification and cognitive decline was shown. We determined the prevalence and investigated the vascular risk factors of hippocampal calcification in patients with cerebrovascular disease.

Methods

Hippocampal calcification was scored bilaterally on presence and severity on CT examinations in a cohort of 1130 patients with (suspected) acute ischemic stroke. Multivariable logistic regression analysis, adjusting for age and gender as well as adjusting for multiple cardiovascular disease risk factors was used to determine risk factors for hippocampal calcification.

Results

Hippocampal calcification was present in 381 (34%) patients. Prevalence increased with age from 8% below 40 to 45% at 80 years and older. In multivariable logistic regression analysis, age per decile (OR 1.41 [95% CI, 1.26-1.57], p<0.01), hypertension (OR 0.74 [95% CI: 0.56-0.99], p=0.049), diabetes mellitus (OR 1.57 [95% CI: 1.10-2.25], p=0.01), and hyperlipidemia (OR 1.63 [95% CI: 1.20-2.22], p<0.01) were significantly associated with hippocampal calcification.

Conclusions

Hippocampal calcification was a frequent finding on CT in this cohort of stroke patients and was independently positively associated with hyperlipidemia and diabetes mellitus, suggesting an atherosclerotic origin.

Introduction

Hippocampal calcification (HC) has only recently been described in vivo for the first time using computed tomography (CT) and was found to be surprisingly common, occurring in up to 20% of subjects over 50 years of age.(1) These calcifications could not be differentiated from the innocuous plexus choroideus calcifications, however, recent advances in CT equipment and reconstruction protocols enabled the differentiation between these two.

To our knowledge just one study on the histology of the hippocampus described calcification of hippocampal vessels and called it vascular fibrosis and calcification (VFC).(2) In this study, VFC was found to be mostly located in the precapillary and capillary arteries in the dentate gyrus and Cornu Ammonis 1 (CA1) of the hippocampal tail and body.(2) On CT examinations HC was observed in the same locations, suggesting a similar origin.(1,3) In the pathology specimens, especially severe HC co-localized with areas of hippocampal neural loss and atrophy and it was suggested that this was due to hypoperfusion because of vascular disease.(2)

In a preliminary study it was shown that HC, as measured with CT, was more common in patients with cognitive problems compared to those without.(3) Although currently still speculative, it maybe that HC and vascular disease in the tail and body of the hippocampus play a role in cognitive decline and dementia. In this light, we were interested in the relation of HC to (potentially modifiable) classic risk factors for vascular disease as they could provide insight into the underlying pathological mechanism of HC and possibly prevention or treatment options. Therefore, given the potential relevance and the limited knowledge on the underlying etiology of HC, we aimed to determine the relation of HC with classic risk factors of vascular disease in a cohort with a high prevalence of vascular risk factors.

Methods

Population

The study population consisted of 1393 patients who were included in the Dutch acute stroke trial (DUST), a prospective multi-center cohort study of (suspected) acute ischemic stroke patients. The DUST study protocol has been published previously.(4) Patients were included from May 2009 until July 2013 in one of six university hospitals and eight non-university hospitals of the Netherlands. All patients over 18 years of age with symptoms of acute ischemic stroke of less than 9-hour duration were included. All patients underwent non-contrast enhanced

CT, CT angiography, and CT perfusion examinations within 9 hours of the start of the symptoms. Patients with known contrast allergy or renal failure were excluded. Specifically for this study we excluded patients for whom no good quality (thin-slice) unenhanced CT was available (n=263). Ethical approval was obtained from the medical ethics committee of the University Medical Center Utrecht, The Netherlands, as well as the local medical ethics committees of the participating centers. All patients or their legal representatives signed informed consent. If a patient died before consent could be obtained, the need for consent was waived by the medical ethics committee.

Baseline measurements

After inclusion, additional patient data were obtained, including age, gender, a medical history of stroke, history of hypertension (systolic blood pressure ≥ 140 mmHg and/or a diastolic blood pressure ≥ 90 mmHg), diabetes mellitus (DM), hyperlipidemia, first-degree family history of cardiovascular disease (CVD) (<60 years of age), smoking (current, former or never), body mass index (BMI), and estimated glomerular filtration rate (eGFR) calculated using the Modification of Diet in Renal Disease (MDRD) formula in μmol/L. An important factor in calculating the eGFR using MDRD is race. As no information on race was available in this cohort and with the majority of the population in the Netherlands being Caucasian, eGFR was calculated assuming all patients were non-black. Analyses were repeated under the assumption that all patients were black but this did not change the results of the analyses. For our analysis, we looked at eGFR and BMI dichotomously, with a threshold of 60 μmol/L for eGFR and 30 for BMI.

Technical information

Multidetector row CT scanners were used, with the number of detectors ranging from 40 to 320 (LightSpeed VCT, GE Healthcare, Milwaukee, Wisconsin; Brilliance 40, Brilliance 64, and Brilliance iCT 256, Philips Healthcare, Best, the Netherlands; Sensation 64, Siemens, Erlangen, Germany; Aquilion ONE, Toshiba Medical Systems, Tokyo, Japan) at 120 kV and 300-375mAs using a standard convolution kernel and an image matrix of 512x512. Patients were scanned from the skull base to the vertex and scans were reconstructed using Filtered Back Projection (FBP) with a slice thickness ranging from 0.625 to 1 mm.

Calcification measurements

The non-contrast enhanced thin slice reconstructions were rated blinded and individually by two experienced radiologists and a medical practitioner with 4, 14, and 2 years of experience in reading (brain) CT scans (J.B.D.V, P.A.d.J and R.K.

respectively). The cohort was divided in equal parts among the three observers. HC is seen in the brain CT window setting (Center: 40, Width: 100) as (clustered) dense configurations comparable to bone located in the hippocampus. HC was scored bilaterally in the hippocampus as absent, mild (one dot), moderate (multiple dots) or severe (confluent) (**Figure 1**).(3) Thin slice reconstructions were used to detect subtle calcifications as they can be easily missed on 5mm thick slice images. To allow for better distinction of mild HC and the increased image noise present on thin slice images, HC was only scored if the calcification was present on two adjacent axial image slices. An example of image noise and mild HC, as scored in this study, is added in the online supplement (**online supplementary figure I**). Additionally, images were analyzed in the coronal and sagittal plane to establish whether a calcification was located within the hippocampus or choroid plexus. For analyzing HC severity, the most severe calcification of either side was used.

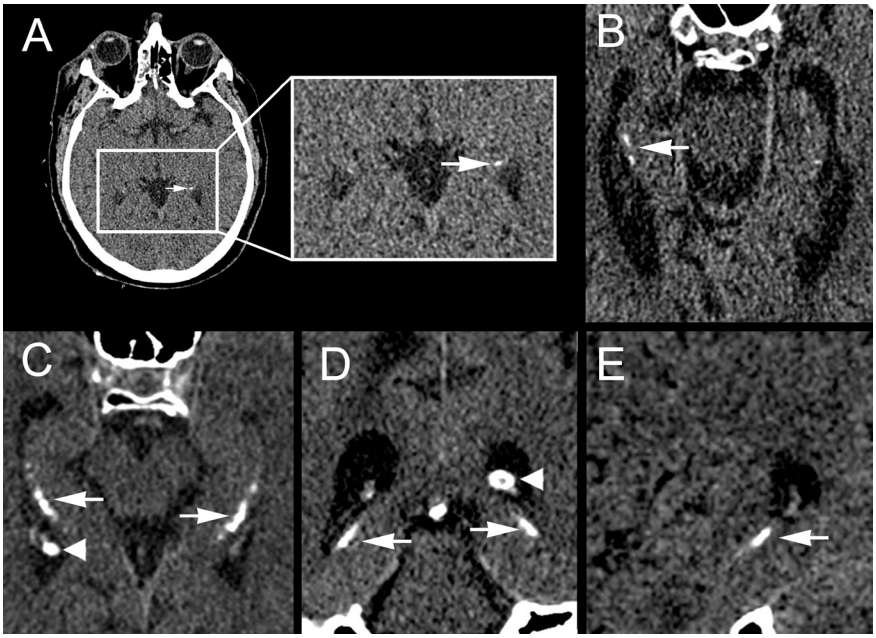

Figure 1 - Hippocampal calcification severity on CT. The severity of hippocampal calcifications (arrows) was scored separately in both the left and right hippocampus as (A) mild (one dot), (B) moderate (multiple dots), and (C-E) severe (confluent calcifications). Calcifications were scored on axial (A-C), coronal (D), and sagittal (E) reconstructions. Calcification of the choroid plexus was also visible (arrowheads).

Statistical analysis

Descriptive statistics were used to describe the characteristics of the study population; means with standard deviations and medians with interquartile ranges (IQR: Q1, Q3) for continuous variables, depending on their distribution, and counts and percentages for categorical variables. To determine differences in baseline values between groups (HC present/absent and severity) chi-squared, Mann-Whitney U, and Analysis of Variance (ANOVA) tests were used for categorical and continuous (parametric/nonparametric) data respectively.

The relation between HC presence and risk factors were studied in multivariable logistic regression analysis. First, we looked at the crude or unadjusted relation between HC and the individual risk factors. Second, we constructed a multivariable model in which we adjusted for age and gender. Lastly, we constructed a multivariable model in which we adjusted for all cardiovascular risk factors: age, gender, history of stroke, hypertension, diabetes mellitus, hyperlipidemia, smoking (current, former, never), BMI (<30 or ≥30), eGFR (<60 or ≥60), and family history of CVD below the age of 60. Multiple imputation using the Markov Chain Monte Carlo method was applied to complete the dataset in case of missing variables (**Table 1**).

Table 1 - Baseline characteristics by hippocampal calcification presence

Characteristic	Total, n=1130 (100%)	HC present, n=381 (33.7%)	HC absent, n= 749 (66.3%)	P-value	Missing %
Age, *yr* (median, IQR)	68.7 (20)	72.7 (16)	67.2 (21)	<.01	0
Gender (male)	643 (56.9)	217 (57.0)	426 (56.9)	.98	0
Stroke in medical history	276 (24.4)	114 (29.9)	162 (21.6)	<.01	0.6
Hypertension	596 (52.7)	208 (54.6)	388 (51.8)	.35	0.9
Diabetes mellitus	171 (15.1)	79 (20.7)	92 (12.3)	<.01	0.4
Hyperlipidemia	388 (35.3)	165 (45.1)	223 (30.5)	<.01	2.8
Smoking					
Current	303 (28.8)	102 (29.1)	201 (28.7)	.43	7
Former	346 (32.9)	123 (35.1)	223 (31.8)		
Never	352 (38.2)	125 (35.7)	227 (39.5)		
Obesity, BMI >30	146 (20.6)	48 (19.5)	98 (21.1)	.61	37
eGFR <60	166 (14.6)	54 (14.2)	112 (15.0)	.85	0.7
1st degree family <60 years with CVD history	245 (32.8)	77 (33.3)	168 (32.6)	.85	34

HC: Hippocampal calcification, IQR: Interquartile Range, CVD: cardiovascular disease, eGFR: estimated glomerular filtration rate (ml/min/1.73m^2, = 32788x(serum creat)$^{-1.154}$ x(age)$^{-0.203}$ x(0.742 if woman)x(1.210 if black)), BMI: Body Mass Index in kg/m^2, data presented as n, %

Interobserver agreement of HC presence (yes/no) and the HC severity score (absent, mild, moderate, severe) was calculated between all three observers in a sample of 50 scans (100 hippocampi) from the cohort using a Fleiss' Kappa and a weighted Kappa (squared) respectively. These 50 scans were randomly distributed through each observers' cohort. A consensus reading determined the final score if there were any discrepancies between the three observers.

Statistical significance was defined as $p<0.05$. Statistical analysis was performed using SPSS (IBM SPSS Statistics, Version 23.0. IBM Corp, Armonk, NY) and R (R Foundation for Statistical Computing, Vienna, Austria.

Results

Characteristics of the study population can be found in **table 1**. The final cohort consisted of 1130 patients of whom the median age was 68.7 years (IQR: 48.7 – 88.7) and 643 (56.9%) were male. A history of hypertension (53%) and hyperlipidemia (35%) as well as a family history of CVD (33%) were highly prevalent. Patients with HC were, on average, older and had more often a history of stroke, DM, and hyperlipidemia.

Hippocampal calcification

Interobserver agreement was good with a Fleiss' Kappa of 0.81 (95% CI: 0.70-0.92) for HC presence and weighted Kappa statistics of 0.91, 0.88, and 0.93 for HC severity between the three observers.

HC was present in 381 (34%) patients and was mild in 188 (17%), moderate in 139 (12%) and severe in 54 (5%). Patient characteristics per severity group can be found in **table 2**. With increasing severity patients were on average older and more frequently had a history of stroke, DM, and hyperlipidemia. Severity per age group is depicted in **figure 2** and showed an increase in percentage of moderate and severe HC with increasing age. Prevalence increased with age from 8% in patients younger than 40 years of age to 45% at 80 years and older. HC was found most frequently bilaterally 223 (58%), followed by unilaterally right 102 (27%), and left 56 (15%) (**Table 3**)

Table 2 - Severity of hippocampal calcification in relation to risk factors

Determinant	Absent n=749	Mild N=188	Moderate N=139	Severe N=54	P-value
Age, yr (median, IQR)	67.2 (21)	69.6 (17)	70.3 (15)	74.0 (17)	<.01
Gender (Male)	426 (56.8)	109 (58.3)	76 (54.7)	32 (59.3)	.92
Stroke in medical history	162 (21.6)	53 (28.3)	40 (29.2)	21 (38.9)	<.01
Hypertension	388 (51.7)	99 (53.2)	77 (55.8)	32 (61.5)	.548
Diabetes mellitus	92 (12.3)	36 (19.3)	32 (23.3)	11 (20.4)	<.01
Hyperlipidemia	223 (30.5)	83 (45.1)	57 (43.5)	25 (49.0)	<.01
Smoking					
Current	201 (28.7)	43 (25.1)	38 (29.5)	21 (42.9)	.22
Former	223 (31.8)	66 (38.4)	42 (32.6)	15 (30.6)	
Never	227 (39.5)	62 (36.6)	49 (38.0)	13 (26.5)	
Obesity, BMI >30	98 (21.1)	32 (24.4)	14 (17.7)	2 (5.6)	.08
eGFR <60	112 (15.0)	20 (14.5)	9 (16.7)	39 (15.2)	.94
1st degree family <60 years with CVD history	168 (32.6)	32 (27.1)	37 (42.0)	8 (32.0)	.16

IQR: Interquartile Range, CVD: cardiovascular disease, eGFR: estimated glomerular filtration rate (ml/min/1.73m2, = 32788x(serum creat)-1.154 x(age)-0.203 x(0.742 if woman)x(1.210 if black)), BMI: Body Mass Index in kg/m2, data presented as n, %

Table 3 - Hippocampal calcification severity in the left and right hippocampus

	HC severity right				
	Absent	Mild	Moderate	Severe	Total
HC severity left					
Absent	749	80	19	3	851
Mild	42	66	31	4	143
Moderate	11	13	65	12	101
Severe	3	3	3	26	35
Total	805	162	118	45	1130

HC: Hippocampal calcification

Uni- and multivariable regression analysis

Results of the uni- and multivariable logistic regression analysis are presented in **table 4**. After adjusting for age and gender; age per decile (OR:1.37, 95%CI: 1.24-1.51, p<0.01, adjusted for gender), history of stroke (OR:1.40, 95%CI: 1.08-1.87, p=0.02), DM (OR:1.68, 95%CI: 1.20-2.35, p<0.01), and hyperlipidemia (OR:1.71, 95%CI:1.31-2.22, p<0.01) were significant.

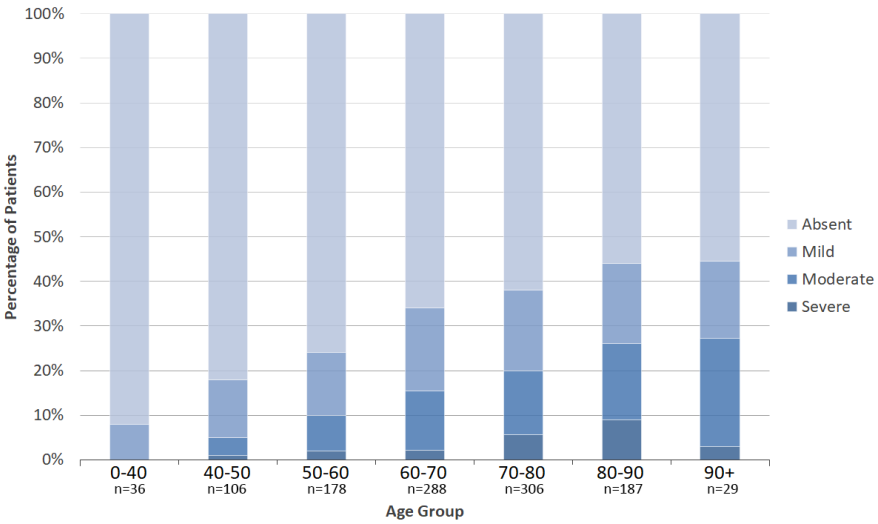

Figure 2 - Hippocampal calcification severity per age group. N = number

Table 4 - Association between risk factors and the presence of hippocampal calcifications

Determinant	Crude OR (95%CI)	P-value	Adjusted OR* (95%CI)	P-value	Multivariable OR (95%CI)	P-value
Age (per decile)	1.35 (1.23-1.49)	.00	1.37 (1.24-1.51)	.00	1.40 (1.26-1.56)	.00
Gender (Male)	1.00 (0.78-1.28)	.98	1.13 (0.88-1.46)	.35	1.04 (0.80-1.36)	.80
Stroke in medical history	1.55 (1.17-2.05)	.00	1.40 (1.05-1.87)	.02	1.21 (0.88-1.64)	.24
Hypertension	1.13 (0.88-1.45)	.34	0.90 (0.69-1.18)	.44	0.74 (0.56-0.99)	.049
Diabetes mellitus	1.86 (1.34-2.59)	.00	1.68 (1.20-2.35)	.00	1.57 (1.10-2.25)	.01
Hyperlipidemia	1.85 (1.43-2.39)	.00	1.71 (1.31-2.22)	.00	1.63 (1.20-2.22)	.00
Smoking						
Current	1.09 (0.79-1.50)	.62	1.35 (0.96-1.91)	.08	1.30 (0.91-1.84)	.15
Former	1.19 (0.87-1.61)	.28	1.17 (0.85-1.61)	.35	1.10 (0.79-1.53)	.59
Never	Reference		Reference		Reference	
Obesity, BMI >30	0.89 (0.63-1.27)	.47	0.96 (0.67-1.36)	.80	0.85 (0.67-1.40)	.97
eGFR <60	0.97 (0.68-1.38)	.72	0.71 (0.49-1.03)	.07	0.72 (0.49-1.05)	.09
1st degree family <60 years with CVD history	1.02 (0.45-1.39)	.99	1.04 (0.75-1.43)	.83	0.99 (0.71-1.39)	.97

* adjusted for age and gender. OR: odds ratio, CVD: cardiovascular disease, eGFR: estimated glomerular filtration rate (ml/min/1.73m^2, = 32788x(serum creat)$^{-1.154}$ x(age)$^{-0.203}$ x(0.742 if woman) x(1.210 if black)), BMI: Body Mass Index in kg/m^2

When adjusting for all CVD risk factors; age per decile (OR: 1.40, 95%CI: 1.26-1.57, p<0.01), hypertension (OR: .74, 95%CI: 0.56-1.00, p=0.049), DM (OR: 1.57, 95%CI: 1.10-2.25, p=0.01), and hyperlipidemia (OR: 1.63, 95%CI: 1.20-2.22, p<0.01) were significant. Additionally, because of the borderline significant OR<1 for hypertension, the effect of treatment with antihypertensive drugs in patients with a history of hypertension was assessed. Treated hypertension remained significant (OR: 0.71, p=0.03) whereas untreated hypertension was no longer significant (OR: 0.78, p=0.27), but the effect size was similar.

Discussion

The main findings of our study are that calcification of the hippocampus is common in a stroke population and increasingly prevalent at higher age. HC was related to several classic cardiovascular atherosclerotic risk factors; age, history of DM, and hyperlipidemia, but not to others; smoking, male gender, CVD history, and renal dysfunction. In multivariate analysis, a weak inverse association with hypertension was found.

From the multivariate analysis, we conclude that HC is independently related to age and history of DM and hyperlipidemia. All of which have been described as risk factors for cardiovascular events, mortality, and arterial calcification in various vascular beds.(5) While older age and DM are related to multiple vascular abnormalities, hyperlipidemia clearly points to atherosclerosis.(6,7) Atherosclerosis is a chronic condition of the intimal layer of the vascular wall which involves accumulation of lipids, inflammation, and calcification and can cause cardio- and neurovascular events.(8) Arterial calcifications often co-localize with these atherosclerotic intimal lesions and are therefore frequently used as a surrogate marker for atherosclerosis on CT images.(9) Other forms of arterial calcification, such as internal elastic lamina calcification and Mönckeberg sclerosis or medial calcification, primarily associate with chronic kidney disease, genetic syndromes, older age, and DM, but not with inflammation and hyperlipidaemia.(10) Therefore, our epidemiological analysis suggests that HC is vascular in origin and most likely atherosclerotic, although further validation is required. Studies looking into the underlying process for HC with histopathological analysis are scarce. Wegiel et al. examined hippocampi of Alzheimer's Disease (AD), Down syndrome and control patients and described a vasculopathy in the walls of hippocampal (pre)capillaries and arteries that they named 'vascular fibrosis and calcification' (VFC).(2) VFC was found to occur in AD patients and age matched controls in 59% and 57% respectively and was shown to cause loss of neurons in the CA1 sector, dentate

gyrus, and subiculum proper through degeneration and occlusion of blood vessels. The most severe form of VFC, progressing to the hippocampal head, appeared to be related to hippocampal sclerosis. Interestingly, Wegiel et al concluded that VFC is not typical for atherosclerosis and that it appeared to be a type of non-atherosclerotic calcification.

What could be the possible consequences of our findings? There are limited data to suggest that HC, possibly as a marker for vascular disease, is related to cognitive decline.(3) HC was most frequently found in the posterior hippocampus in both CA1 and the dentate gyrus. These hippocampal regions have been related to AD, vascular disease, and ageing. HC could therefore play a role in neurodegenerative, vascular, and age related hippocampal disease.(11,12) A possible relation to AD, however, appears less likely because even though HC was frequently found in patients with AD, Wegiel et al. reported that only 4% of patients with down syndrome showed VFC while 96% were affected by AD, opposing a common pathological mechanism.(2) While AD and HC might have a combined effect on hippocampal function and cognition, distinction between both pathologies can be of importance to measure preventive and treatment interventions. Risk factors for HC identified in our study could provide a starting point for treatment as both DM and hyperlipidemia are modifiable. First and foremost through prevention and secondly, through lifestyle improvements like treatment with lipid lowering drugs, glucose stabilization, and exercise. High levels of blood glucose and DM2 have been related to metabolic defects within the hippocampus and hippocampal atrophy.(12,13) The beneficial effects of exercise and fitness on hippocampal volume and cognition has been described and treatment with cholesterol lowering therapy showed slowing of cognitive decline and atrophy of both medial temporal lobes.(14,15)

The presence and severity of HC in this cohort appears mostly symmetrical, still, some dissimilarities are present. HC was found more often unilaterally right, which was also described in other studies, and was on average slightly more severe on that side.(3) The bilateral function of the hippocampus is largely unknown, however, evidence of hippocampal lateralization has been reported.(16,17) In cases of unilateral hippocampal atrophy both the maintaining as well as decline of cognitive function has been described.(18,19)

After correcting for other CVD risk factors we found a weak inverse relation of 'history of hypertension' with HC even though there was a higher prevalence of HC in these patients. A good explanation here is lacking and it points at the possiblelimitations of our study. First, the cohort in which this study was

performed consisted entirely of patients with (suspected) stroke without a healthy reference sample. This limits the generalizability of the prevalence of HC to the general population and some caution with the risk factors is warranted, especially on the preventative effect of hypertension. Second, HC was scored on thin-slice images which are more susceptible to image noise compared to regular/thick slice images. The rationale for this is that subtle HC can easily be missed on 5mm slices. While the use of thin slice images can increase false positive findings, we are confident that the distinction can be made adequately with the methods described in our study. Third, data from a multicenter study was used with different CT-scanners. However, all CT-scanners were state-of-the-art with good quality CT scans and we think this did not influence our visual scores. Fourth, HC was scored categorically and not volumetrically. The categorical score was preferred as it is fast and reproducible and volumetric measurement based on a density/HU threshold of 130 could prove difficult due to the adjacent choroid plexus and image noise on thin-slice data. Furthermore, dichotomous logistic regression analysis could now be performed, providing odds ratios which are often better understandable for readers/physicians. Fifth, information on BMI and family history were missing for more than 30% of the patients. We imputed the data using multiple imputation to allow for optimal multivariable analysis. Finally, to assess HC in this sizable cohort, it was divided and scored by three observers showing a good interobserver agreement (Fleiss' Kappa: 0.81). The advantage of this approach was that we were able to investigate a large cohort with reasonable effort. However, our approach might have introduced some level of error in the prevalence estimation and associations compared to a situation where multiple observers would have scored all images with a subsequent consensus reading to solve discrepancies. Based on the blinded analysis and good agreement between observers, the misclassification is likely non-differential and therefore unrelated to the investigated risk factors. While non-differential misclassification can cause overestimation, it often results in an underestimation of the reported associations.(20)

To conclude, HC is a frequent finding in a cohort of patients suspected of acute stroke and is related to modifiable cardiovascular risk factors such as hyperlipidemia and DM. Still, our findings need to be confirmed and further radiology-pathology and radiology-outcome association studies on this subject are needed. This may lead to a new strategy for prevention and treatment of these modifiable risk factors of HC and associated cognitive decline.

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CHAPTER 8

Hippocampal calcifications: risk factors and association with cognitive function

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Abstract

Background

Our aim was to identify risk factors for hippocampal calcifications and to investigate the association of hippocampal calcification with cognitive function.

Methods

For this retrospective study, consecutive patients visiting a memory clinic at a Dutch general hospital between April 2009 and April 2015 were identified. All individuals underwent a standard diagnostic work-up including cognitive tests and brain CT scan. The following vascular risk factors were assessed: hypertension, diabetes mellitus, hyperlipidemia and smoking. Cognitive screening consisted of the Cambridge Cognitive Examination, which includes the Mini Mental State Examination. CT scans were analyzed for presence and severity (absent, mild, moderate, severe) of hippocampal calcifications. One measure per patient, only the most severe score, was used. We used logistic regression to identify risk factors for hippocampal calcifications and linear regression for the association between hippocampal calcifications (patient level) and cognitive function.

Results

A total of 1991 patients (mean age 78 years (females 79 vs males 77), range 45;96, (females 47;96 vs males 45;95)) were included, of whom 380 (19.1%) had hippocampal calcifications. Older age (odds ratio [OR] per year 1.05, 95% confidence interval [CI] 1.03-1.06), diabetes mellitus (OR 1.50, 95% CI 1.12-2.00) and smoking (OR 1.49, 95% CI 1.05-2.10) were associated with the presence of hippocampal calcifications. No associations were found between presence and severity of hippocampal calcification and cognitive function.

Conclusion

Older age, diabetes mellitus and smoking were associated with an increased risk of hippocampal calcifications. Greater degree of hippocampal calcifications was not associated with lower cognitive function in patients with memory complaints.

Introduction

Dementia is a substantial health problem with 46.8 million people suffering from this condition worldwide.(1) Dementia mostly appears to result from a combination of factors, including Alzheimer disease (AD), vascular lesions, Lewy bodies and inflammation, which eventually leads to atrophy of the cortex and hippocampus.(2) The hippocampus is an important area of interest in dementia research. Current research into hippocampal pathology in dementia focuses more on neurodegenerative etiologies and less on vascular causes. Hippocampal calcifications were first described in a pathology study in 2002 as a vasculopathy with fibrosis and calcification with a predilection for the middle hippocampal artery (3). These calcifications can spread from the tail to the body of the hippocampus, and occasionally to the head, and may lead to patchy neuronal loss. Accordingly, it has been hypothesized that hippocampal calcifications may be a manifestation of vascular pathology that could contribute to hippocampal atrophy and consequently cognitive deterioration.(3)

Advances in radiologic imaging have provided opportunities to explore the role of hippocampal calcifications in dementia. In a study using multiplanar brain computer tomography (CT) scans, hippocampal calcifications were frequently observed and appeared to increase with age, with hippocampal calcifications being detected in more than 20% of individuals above 50 years old.(4) Due to their proximity, hippocampal calcifications may be difficult to distinguish from plexus calcifications and prior to multiplanar CT, hippocampal calcification may have been mistaken for choroidal calcification. The literature on the association between hippocampal calcification and cognitive impairment is limited to a small case-control study, in which hippocampal calcifications were found more often in patients from a memory clinic than in matched controls. Moreover, individuals with hippocampal calcifications were found to have a lower Mini Mental State Examination (MMSE) score.(5)

We hypothesized that patients with hippocampal calcifications are more likely to have vascular risk factors and that hippocampal calcifications are associated with lower scores on cognitive tests.

The aim of our study was to identify vascular risk factors for hippocampal calcifications in a large cohort of individuals visiting an outpatient memory clinic. Furthermore, we aimed to evaluate the clinical importance of hippocampal calcifications by studying the association of hippocampal calcification with cognitive function.

Methods

Study population

Our retrospective cross-sectional study consisted of 2,000 consecutive patients who visited the memory clinic of Tergooi hospital, a general hospital in the Netherlands, between April 2009 and April 2015. In a previously published article, all 2,000 patients were included and the prevalence of mixed dementia in patients with late-onset AD was investigated.(6) In this manuscript, we report the risk factors of hippocampal calcifications and the association with cognitive function.

The inclusion criteria for this study was referral to the memory clinic of Tergooi hospital between April 2009 and April 2015 because of memory complaints. All patients underwent a standard diagnostic work-up, including a brain CT scan. The only exclusion criterion for our study was absence of a brain CT scan, which led to exclusion of nine patients. None of the CT scans had to be excluded because of motion artifacts. Of the 1,991 remaining patients, 40.3 % were male and the mean age was 78 years (range 45;96).

The local Medical Ethics Committee of Tergooi hospital gave approval for our study and waived the need for informed consent.

Diagnostic procedures

All patients referred to the memory clinic underwent a standard diagnostic work-up including history-taking, medical and neurological examinations, assessment of vital functions, assessment of education level according to Verhage (details in the supplement)(7), cognitive screening including the Cambridge Cognitive Examination (CAMCOG) which contains the MMSE (full details in the supplement) (8,9), electrocardiogram, laboratory tests, head CT scan and history taking with a relative or other acquaintance. The CAMCOG is a well validated cognitive evaluation and includes the most important cognitive domains that might be impaired in dementia and has a high sensitivity and specificity (92% and 96%, respectively) for detecting cognitive decline.(8,9) Vascular risk factors including hypertension, diabetes mellitus (DM), hyperlipidemia and smoking were assessed during history-taking (yes/no). Smoking was classified into smoking, or non smoking (also including previous smoking). Finally, a diagnosis was established, including mild cognitive impairment, AD, or Vascular Dementia, according to the standard clinical diagnostic criteria (10,11,12) (further details are in the supplement).

Computed tomography protocol

All patients underwent a brain CT scan. A 64-detector row CT (Siemens Somatom Definition AS, Siemens Healthineers, Erlangen, Germany) was used to scan patients from the base of the skull to the vertex. The acquisition parameters were 120 kV, 260 mAs, 64 * 0.6mm collimation, pitch of 0.55, WC / WW=40 / 80, CARE kV=on (dose optimation slider on noncontrast). Scans were reconstructed as oblique coronal slices of 3.0 mm, axial slices of 5 mm with soft tissue window and 1.5 mm axial slices in bone window.

Hippocampal calcification

CT scans were analyzed for hippocampal calcifications by one medical doctor (either EdB or RK) who was blinded for all risk factors, except age and gender, and blinded for the clinical outcomes. All CT scans were analyzed in a time span of two weeks. One medical doctor has 5 years of clinical experience in geriatrics (EdB). The other medical doctor has 3 years of experience in assessing CT images during his PhD program (RK). These investigators were trained by a vascular radiologist (PdJ, 10 years of experience) and by a neuroradiologist (JH, 15 years of experience). Images were analyzed in axial and coronal planes in the brain window setting (Center: 40, Width: 100) using a previously established score.(5) Calcifications were scored by presence and severity: absent, mild (one dot)(**Figure 1**), moderate (multiple dots)(**Figure 2**) or severe (confluent)(**Figure 3**). Both hippocampi were scored separately. When analyzing for severity, the most severe calcification score, either for right or left, was used. When analyzing for presence of hippocampal calcifications, hippocampal calcification was considered present if a calcification was scored in at least one hippocampus. In this way, we used only one measure for hippocampal calcification presence and one measure for hippocampal calcification severity per patient.

Statistical analysis

Descriptive statistics were used to summarize the baseline characteristics. The Chi-squared test (for trend) and Mann-Whitney U test were performed to compare categorical and continuous variables, respectively, between individuals with and without hippocampal calcification. Logistic regression was performed to identify risk factors (explanatory variables) associated with the presence of hippocampal calcifications (outcome variable), including univariable analyses (model 1), age- and sex adjusted analyses (model 2), and analyses adjusted for age, sex, hypertension, DM, hyperlipidemia and smoking status (model 3). The Chi-squared test was used to analyze whether number of risk factors (age >80,

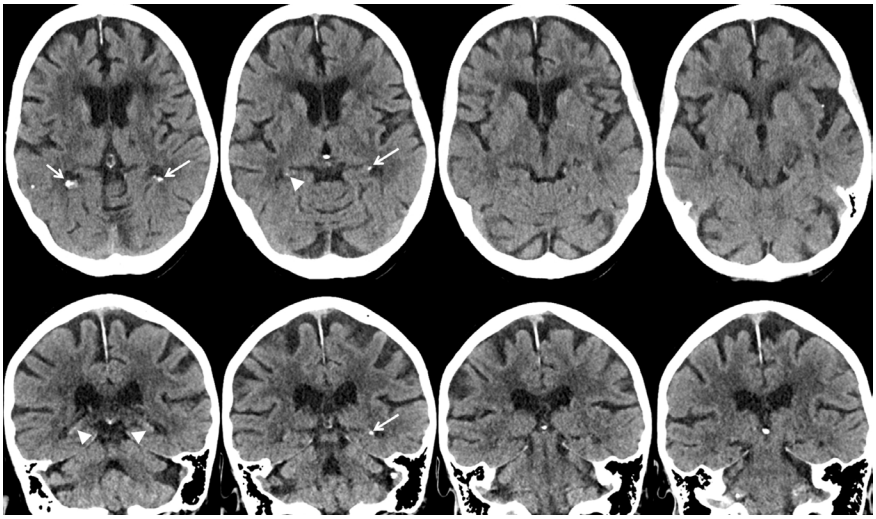

Figure 1 - Mild hippocampal calcification on CT examination (arrowheads) of an 88-year old female. The image shows mild hippocampal calcification (arrowheads). The arrows show calcification of the choroid plexus.

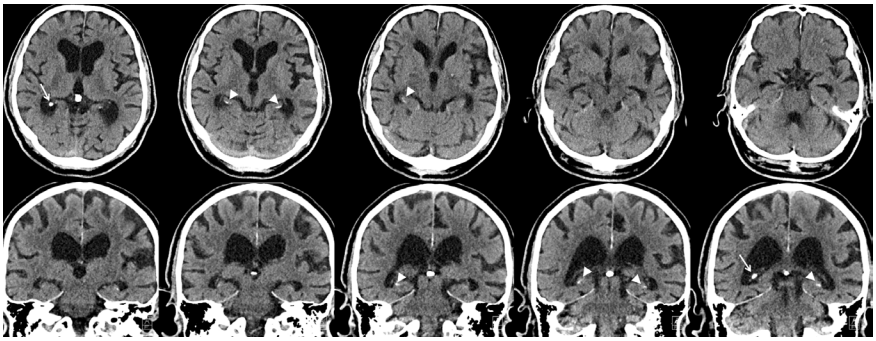

Figure 2 - Moderate hippocampal calcification on CT examination (arrowheads) of a 74-year old female. The image shows mild hippocampal calcification (arrowheads). The arrows show calcification of the choroid plexus

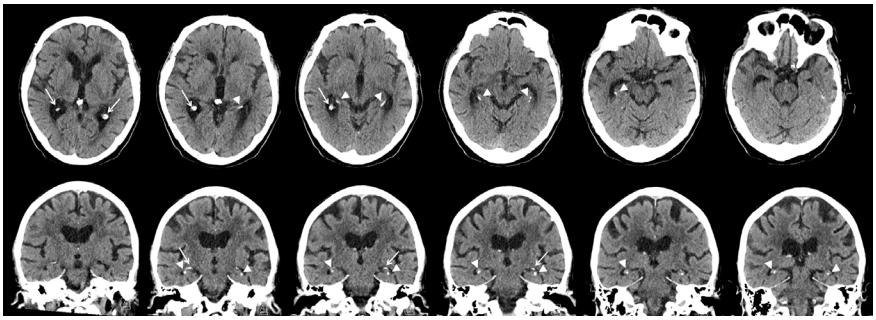

Figure 3 - Severe hippocampal calcification on CT examination (arrowheads) of a 76-year old man. The image shows mild hippocampal calcification (arrowheads). The arrows show calcification of the choroid plexus.

DM and smoking) was associated with severity of hippocampal calcifications. Linear regression was used to analyze if hippocampal calcification (explanatory variable) is associated with cognitive function (outcome variable). A reflect and square root transformation (√((largest value of outcome variable + 1) – original value of outcome variable)) was used to obtain a normal distribution, checked with the Q-Q plot, of the CAMCOG and MMSE outcomes. In the results section under the heading 'cognitive outcomes', beta's obtained after transformation of the outcome variables (MMSE or CAMCOG) to a normal distribution are presented. Univariable analyses (model 1), age- and sex adjusted analyses (model 2), and analysis adjusted for age, gender and education level (model 3) were performed. In addition, subgroup analyses were performed for patients with either mild cognitive impairment, AD, or Vascular Dementia. Patients with missing values on the cognitive tests were excluded from the regression analyses.

A two-sided p-value below 0.05 was considered to be statistically significant. Cohen's kappa was used to estimate the interobserver agreement of hippocampal calcification presence (yes/no) in a sample of 50 CT scans. Statistical analyses were performed using SPSS version 24 (IBM Corp., Armonk, New York).

Results

Patients

The cohort existed of 2,000 patients. Nine patients were excluded from the analysis because there was no CT scan of the brain present. This resulted in a total of 1,991 patients. Of the 1,991 patients, 380 (19.1%) had hippocampal calcifications (**Table 1**). The interobserver agreement was good (kappa coefficient of 0.80 and 0.78 when weighted for severity scores).

Risk factors for hippocampal calcification

Before correcting for confounders, we found that patients with hippocampal calcification were older than those without hippocampal calcification (mean 81 vs 78 years, p-value <0.01) and had a lower education level than patients without hippocampal calcification (mean 4.20 vs 4.37, p-value 0.05). More patients with hippocampal calcification had a history of DM (20.8% vs 14.8%, p-value 0.01) (**Table 1**). Other vascular risk factors were similarly prevalent among both groups. When corrected for confounders, we found that older age (OR 1.05, 95% CI 1.03-1.06, p-value < 0.01), DM (OR 1.50, 95% CI 1.12-2.00, p-value < 0.01) and smoking (OR 1.49, 95% CI 1.05-2.10, p- value 0.02) were associated with the presence of hippocampal calcification (**Table 2**, model 2).

Table 1 - Baseline characteristics by hippocampal calcification presence

	Total, n= 1991 (100%)	HC present, n= 380 (19.1%)	HC absent, n= 1611 (80.9%)	P-value
Age (yrs), mean (IQR)	78 (45;96)	81 (62;95)	78 (45;96)	**<0.01**
Gender, male (%)	802 (40.3)	164 (43.2)	638 (39.6)	**0.22**
Education level, mean (SD)	4.34 (1.5)	4.20 (1.5)	4.37 (1.5)	**0.05**
Cerebral infarct, n (%)	104 (5.2)	13 (3.4)	91 (5.6)	**0.10**
TIA, n (%)	116 (5.8)	25 (6.6)	91 (5.6)	**0.47**
Hypertension, n (%)	675 (33.9)	129 (33.9)	546 (33.9)	**1.00**
Diabetes mellitus, n (%)	317 (15.9)	79 (20.8)	238 (14.8)	**0.01**
Hyperlipidemia, n (%)	153 (7.7)	27 (7.1)	126 (7.8)	**0.75**
Smoking, n (%)	228 (11.5)	51 (13.4)	177 (11.0)	**0.18**
MMSE, median (IQR)	23 (18;26)	22 (18;26)	23 (18;26)	**0.39**
CAMCOG, median (IQR)	76 (63;85)	75 (61;84)	76 (63;86)	**0.04**

Age, MMSE, CAMCOG, analysed with Mann-Whitney U test, other characteristics analysed with Chi-square test, education with the Chi-square test for trend. n: number, HC: hippocampal calcification, IQR: Interquartile Range, yrs: years, SD: standard deviation, MMSE: Mini Mental State Examination, CAMCOG: Cambridge Cognitive Examination, TIA: Transient Ischemic Attack.

Table 2 - Risk factors for presence of hippocampal calcification

Risk factors	Univariable analysis			Age/sex adjusted analysis			Multivariable analysis		
	Model 1			Model 2			Model 3		
	OR	CI	P	OR	CI	P	OR	CI	P
Age	1.04	1.03-1.06	<0.01	1.05	1.03-1.06	<0.01	1.05	1.03-1.06	<0.01
Gender	0.86	0.69-1.08	0.20	0.78	0.62-0.98	0.03	0.81	0.64-1.02	0.07
Hypertension	1.00	0.79-1.27	0.98	0.95	0.75-1.21	0.68	0.91	0.71-1.17	0.47
Diabetes Mellitus	1.51	1.14-2.01	<0.01	1.47	1.10-1.95	<0.01	1.50	1.12-2.00	<0.01
Smoking	1.25	0.90-1.75	0.18	1.50	1.06-2.11	0.02	1.49	1.05-2.10	0.02
Hyperlipidaemia	0.90	0.59-1.39	0.64	0.91	0.59-1.41	0.67	0.89	0.57-1.39	0.60
Education level	0.93	0.86-1.00	0.05	0.94	0.87-1.01	0.10	0.95	0.88-1.02	0.17

Data analysed with logistic regression. Multivariable analysis adjusted for age, gender, hypertension, Diabetes Mellitus, smoking and hyperlipidaemia. OR: Odds ratio, CI: confidence interval.

Education level was not associated with presence of hippocampal calcification anymore (OR 0.95, 95% CI 0.88-1.02, p-value 0.17). Hypertension (OR 0.91, 95% CI 0.71-1.17, p-value 0.47) and hyperlipidemia (OR 0.89, 95% CI 0.57-1.39, p-value 0.60) were not associated with a higher risk of hippocampal calcification. Number of risk factors (age > 80 years, DM, smoking) was associated with the severity of hippocampal calcifications ($p < 0.01$). In patients without hippocampal calcifications or mild calcifications 11.1 % had 2 risk factors and 0.5% had 3 risk factors. In patients with moderate or severe calcifications 17.0% had 2 risk factors and 3.1% had 3 risk factors (**Table 3**).

Table 3 - Number of risk factors and severity of hippocampal calcification

Number of risk factors	Severity of hippocampal calcification				Total
	No HC	Mild HC	Moderate HC	Severe HC	
0, n (%)	600 (85.2)	61 (8.7)	32 (4.5)	11 (1.6)	704 (100)
1, n (%)	840 (80.7)	117 (11.2)	59 (5.7)	25 (2.4)	1041 (100)
2, n (%)	160 (69.6)	43 (18.7)	22 (9.6)	5 (2.2)	230 (100)
3, n (%)	9 (64.3)	0 (0.0)	3 (21.4)	2 (14.3)	14 (100)
Total, n (%)	704 (35.4)	1041 (52.3)	230 (11.6)	14 (0.7)	1989 (100)

P-value <0.01. Analyzed with Chi-squared test. Risk factors are age >80 years, diabetes mellitus and smoking. HC: hippocampal calcification, n: number.

Cognitive outcomes

The majority of patients in our cohort were diagnosed with AD (41.6%), followed by mild cognitive impairment (24.6%) and subjective cognitive impairment (16.7%) (**Supplemental table 1**). Less than 1% of patients had missing values regarding the CAMCOG and MMSE.

Uncorrected for confounders, patients with hippocampal calcification had a lower score on the total CAMCOG (median 75 vs 76, p-value 0.04). After correction for confounders, we did not find differences in outcomes of the CAMCOG (beta -0.05, p-value 0.48) and MMSE (beta -0.08, p-value 0.09) between patients with and without hippocampal calcification (**Table 4**). There were also no differences in cognitive function when analyzed according to severity (p-values 0.37 (CAMCOG) and 0.07 (MMSE))(**Table 4**). Analysis according to subgroups of patients with mild cognitive impairment, AD, or Vascular Dementia gave the same results (p-values ranging from 0.11-0.67) (**Supplemental tables 2, 3, 4**).

Table 4 - Association between hippocampal calcification and cognition

Cognitive tests	Univariable analysis			Age/sex adjusted analysis			Multivariable analysis		
	Model 1			Model 2			Model 3		
	Beta	CI	P	Beta	CI	P	Beta	CI	P
Hippocampal calcification presence									
CAMCOG	0.18	0.00-0.35	0.05	-0.00	-0.16-0.16	0.99	-0.05	-0.20-0.10	0.48
MMSE	0.05	-0.05-0.16	0.33	-0.06	-0.16-0.05	0.28	-0.08	-0.18-0.01	0.09
Hippocampal calcification severity									
CAMCOG	0.05	-0.05-0.15	0.32	-0.02	-0.11-0.08	0.72	-0.04	-0.13-0.05	0.37
MMSE	0.00	-0.06-0.07	0.93	-0.04	-0.10-0.02	0.17	-0.05	-0.11-0.00	0.07

Data analysed with linear regression. Multivariable analysis adjusted for age, gender and education level. CI: confidence interval, CAMCOG: Cambridge Cognitive Examination, MMSE: Mini Mental State Examination.

Discussion

Our cross-sectional study in memory clinic patients showed that older age, DM and smoking were associated with the presence of hippocampal calcifications. Presence and higher severity of hippocampal calcification were not associated with lower cognitive function in our cohort of memory clinic patients, which included patients with subjective cognitive impairment, mild cognitive impairment, dementia due to AD and vascular dementia.

There currently are limited data on risk factors for hippocampal calcifications. To our knowledge, only three previous articles have been published on hippocampal calcifications.(3,4,5) The prevalence of hippocampal calcification in our study was 19.1%, which is comparable to the 21.7% observed in an Australian university hospital of 217 randomly selected CT scans of patients older than 50 years of age, although another study observed a higher prevalence of 38.8% in 67 patients visiting a memory clinic and a prevalence of 13.4% in the control group.(4,5) Both studies also used multiplanar CT scans.(4,5) All studies showed a strong association of hippocampal calcification with older age. Our study is consistent with this finding. One previous study assessed the association of vascular risk factors with hippocampal calcification.(4) There was no significant difference in the number of vascular risk factors in patients with hippocampal calcification and without hippocampal calcification. However, in this previous study, the sample of 47 patients with hippocampal calcification compared with 253 patients without hippocampal calcifications was too small to draw any firm conclusions.(4) In our study, we show an association of hippocampal calcification with DM and smoking, but not with other vascular risk factors. Therefore hippocampal calcification could be a marker of vascular pathology. Wegiel et al. describe in their pathology study a non-arteriosclerotic process of vasculopathy with fibrosis and calcification.(3) The lack of association with hypertension and dyslipidemia may support non-atherosclerotic arterial calcifications, although this remains speculative until further verification by histology. Our findings support that these calcifications may be of vascular origin. Currently, if radiologists decide to report hippocampal calcifications in a radiology report, we suggest reporting the presence of hippocampal calcification as mild/moderate/severe (vascular) calcifications in the hippocampus on the right/left side, with the remark that this is an incidental finding of currently unknown clinical relevance.

Given the location of hippocampal calcifications in the hippocampal tail and the association with some vascular risk factors, we hypothesized that hippocampal calcifications could be associated with cognitive decline. A pilot study in patients

visiting a memory clinic observed hippocampal calcifications three times more commonly in these patients compared to controls and reported that hippocampal calcifications was associated with lower MMSE scores.(5) In our study, however, we did not find a significantly lower score on any of the cognitive tests in patients with hippocampal calcifications, including the MMSE. A possible explanation for this discrepancy is the correction for age, gender and education level in our study, while the pilot study did not correct for these potential confounding factors. Hippocampal calcification does not seem to be associated with cognitive decline Further population-based cohort studies are needed to confirm our findings.

There are limitations of our study that need to be taken into account when interpreting the data. First, there is a referral bias because all patients were referred to a memory clinic. Although some patients did not have cognitive impairment on examination, they did have memory complaints for which they were referred. We did not have a control group of persons without memory complaints, which might have led to an underestimation of the association between hippocampal calcification and cognition. Thin slice reconstructions of the CT scans were unavailable, which could have led to an underestimation of the presence of hippocampal calcifications by missing subtle calcifications. However, if hippocampal calcifications had an effect on cognitive function, we would expect to find this with the more severe calcifications and not with the subtle calcifications we might have missed. Another limitation is the measurement of cardiovascular risk factors. The presence of cardiovascular risk factors was based on history taking. This may have led to misclassification, especially in patients with memory complaints, as some patients classified as not having a cardiovascular risk factor actually did have the risk factor when determined by laboratory tests or presence of medication. This might have led to an underestimation of the associations that were found between the risk factors and the presence of hippocampal calcifications. Additionally, the smoking classification was arbitrary, as non smokers may have stopped smoking recently. This could have led to an overestimation of the risk of smoking.

In conclusion, older age, DM and smoking appear to be associated with an increased risk of hippocampal calcifications on CT, which suggests that these calcifications may be of vascular origin. The findings from our study do not support hippocampal calcifications as a marker of cognitive impairment in patients visiting a memory clinic, although further confirmation is needed.

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Supplemental Information

Details of diagnostic procedure

Education levels were classified according to Verhage, a Dutch classification system including 7 categories; 1= did not finish primary school, 2= finished primary school, 3= did not finish secondary school, 4= finished secondary school, low level, 5= finished secondary school, medium level, 6= finished secondary school, highest level, and/or college degree, 7= university degree.(1)

Cognitive screening consisted of the Cambridge Cognitive Examination (CAMCOG) (part B of the Cambridge Examination for Mental Disorders). The CAMCOG consists of 67 items with a maximum score of 107 and can be divided into the following subscales: orientation, expressive and comprehensive language, memory (remote, recent and learning), attention, praxis, calculation, abstraction and perception.(2,3) All items of the MMSE are incorporated into the CAMCOG.

Following the diagnostic work-up, a consensus meeting with a geriatrician, neurologist, neuropsychologist and a specialized nurse took place, during which a diagnosis was made. A diagnosis of Mild Cognitive Impairment, AD and Vascular Dementia was made according to the standard clinical diagnostic criteria at that time.(4, 5, 6) Patients were diagnosed with other forms of dementia (including Frontotemporal Dementia, Parkinson Dementia, Dementia with Lewy Bodies), or other diagnoses (i.e., a neurologic or psychiatric disorder) also according to the guidelines at that time.(7, 8, 9) If cognitive testing was normal and there was no sign of an underlying psychiatric or neurologic disorder to explain the memory complaints, patients were diagnosed with subjective cognitive impairment.

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Supplemental table 1 - Diagnosis by hippocampal calcification presence

Diagnosis	Total, n= 1991 (100%)	HC present, n= 380 (19.1%)	HC absent, n= 1611 (80.9%)	P-value
SCI, n (%)	332 (16.7)	55 (14.5)	277 (17.2)	0.22
MCI, n (%)	490 (24.6)	91 (23.9)	399 (24.8)	0.79
AD, n (%)	829 (41.6)	173 (45.5)	656 (40.7)	0.09
VaD, n (%)	56 (2.8)	11 (2.9)	45 (2.8)	0.86
Other dementia, n (%)	53 (2.7)	7 (1.8)	46 (2.9)	0.37
Other diagnosis, n (%)	231 (11.6)	43 (11.3)	188 (11.7)	0.93

Data analyzed with Chi-square test. SCI: Subjective Cognitive Impairment, MCI: Mild Cognitive Impairment, AD: Alzheimer's Disease, VaD: Vascular Dementia, n: number.

Supplemental table 2 - Association between presence of hippocampal calcification and cognition in patients with Mild Cognitive Impairment

Cognitive tests	Univariable analysis			Age/sex adjusted analysis			Multivariable analysis		
	Model 1			Model 2			Model 3		
	Beta	CI	P	Beta	CI	P	Beta	CI	P
CAMCOG	-0.02	-0.24-0.21	0.90	-0.02	-0.23-0.19	0.85	-0.07	-0.27-0.12	0.46
MMSE	-0.02	-0.17-0.13	0.80	-0.03	-0.18-0.12	0.70	-0.06	-0.20-0.09	0.43

Data analysed with linear regression. Multivariable analysis adjusted for age, gender and education level. CI: confidence interval, CAMCOG: Cambridge Cognitive Examination, MMSE: Mini Mental State Examination.

Supplemental table 3 - Association between presence of hippocampal calcification and cognition in patients with Alzheimer disease

Cognitive tests	Univariable analysis			Age/sex adjusted analysis			Multivariable analysis		
	Model 1			Model 2			Model 3		
	Beta	CI	P	Beta	CI	P	Beta	CI	P
CAMCOG	0.12	-0.08-0.32	0.25	0.10	-0.10-0.30	0.31	0.06	-0.13-0.24	0.54
MMSE	0.00	-0.12-0.12	0.98	-0.00	-0.13-0.12	0.95	-0.03	-0.14-0.09	0.67

Data analysed with linear regression. Multivariable analysis adjusted for age, gender and education level. CI: confidence interval, CAMCOG: Cambridge Cognitive Examination, MMSE: Mini Mental State Examination.

Supplemental table 4 - Association between presence of hippocampal calcification and cognition in patients with Vascular Dementia

Cognitive tests	Univariable analysis			Age/sex adjusted analysis			Multivariable analysis		
	Model 1			Model 2			Model 3		
	Beta	CI	P	Beta	CI	P	Beta	CI	P
CAMCOG	-0.70	-1.43-0.03	0.06	-0.68	-1.37-0.02	0.06	-0.50	-1.19-0.19	0.15
MMSE	-0.52	-1.04-0.00	0.05	-0.52	-1.05-0.00	0.05	-0.43	-0.96-0.10	0.11

Data analysed with linear regression. Multivariable analysis adjusted for age, gender and education level. CI: confidence interval, CAMCOG: Cambridge Cognitive Examination, MMSE: Mini Mental State Examination.

CHAPTER 9

General Discussion

Intracranial arterial calcification is a frequent finding on computed tomography (CT) scans of the brain and is increasingly recognised as a risk-factor for cerebrovascular diseases. The aim of this thesis was to increase knowledge on these calcifications. First, we wanted to know what these calcifications represent and where they are located. For this, CT imaging findings were validated with histopathology. While this is a labour-intensive process, it prevents spurious assumptions and conclusions that are common in cardiovascular medicine. Subsequently the relation of intracranial arterial calcifications with established cardiovascular risk factors was investigated, and a lifespan prevalence study was performed. In this thesis, we focused on calcifications in the intracranial carotid artery and in the hippocampus and will discuss both calcifications separately. Finally, we propose how to continue in this increasingly relevant medical field.

Novel insights into Intracranial Carotid Artery Calcification

Intracranial carotid artery calcification (ICAC), is one of the strongest risk factors for ischemic cerebrovascular disease, reported to contribute to up to 75% of all strokes.(1) Intracranial calcification can lead to stroke via thromboembolism, in-situ thrombotic occlusion, and hemodynamic impairment through stenosis and arterial stiffening.(2,3) The risks of cerebral atherosclerotic diseases are increasingly understood and enormous effort is put into prevention and treatment. Despite these efforts, projections by the American Heart Association show that by 2030 prevalence of stroke will rise by 20.5%.(4) This is most likely due to the aging population since age is by far the most important non-modifiable risk factor. Since ICAC increases strongly in prevalence and severity after 40 years of age, further understanding of ICAC can provide additional insight into the etiology and possibly new or better targeted therapeutic options for the aging population. In this paragraph, we will discuss three of our main findings about ICAC:

1. ICAC is composed of two types of calcification which can be distinguished on CT
2. These two different types of calcification have a different risk profile
3. ICAC can already be seen in young children and adolescents

1. It was previously assumed that ICAC is a form of atherosclerosis.(5,6) and that the calcifications represented calcified atherosclerotic lesions. Research from our group recently demonstrated that both intimal and medial calcifications can be present and that medial calcification predominated in 70% of intracranial carotid artery specimens.(5) The ability to differentiate between intimal and medial calcification allows for independent investigation of the meaning of these

two different types of calcification. Thereby, intimal disease is expected to relate to acute events through thromboembolic events, stenosis and plaque ruptures whereas medial calcification may have a more chronic effect through decreased arterial elasticity and remodelling resulting in increased pulse pressure and damage to small arteries.(7,8) Increased arterial stiffness is known to be an independent risk factor for cardiovascular disease and has been related to stroke and dementia.(7–9) Direct distinction between calcifications found in the two adjacent arterial wall layers is currently not possible in clinical imaging since these layers are too thin to be identified with state of the art imaging methods, such as transcutaneous ultrasound, (ultra-high field) Magnetic Resonance Imaging (MRI) or CT. The ICAC score that we developed for routine unenhanced CT is a first step into providing an in vivo imaging method to distinguish the two major calcification types in the intracranial carotid artery. The main limitation of the score is that it determines calcification dominance and not the actual intimal or medial calcification presence. This is largely because, in the vast majority of patients, both types of ICAC are present and overlap exists. For example, when dominant medial calcification is determined by the score it is still possible that some intimal calcification is also present. Considering this, the score was still able to discern two distinct risk profiles for intimal and medial calcification in the intracranial carotid artery. Finally, even though a methodology to score the ICAC characteristics are provided and visual examples are available, the score remains observer based and therefore subject to bias. Possibly, computer assisted detection, as is already applied to coronary artery calcification, could improve the score and negate potential observer bias.

2. Medial calcification in the internal carotid artery was common in a large cohort of cerebrovascular patients and had different risk factors compared to intimal calcification. In the Dutch acute Stroke Trial (DUST) age, pulse pressure and family history of cardiovascular disease were risk factors for both types of ICAC. Risk factors for only dominant intima calcifications were smoking and hypertension whereas diabetes mellitus and previous vascular disease were related to dominant medial calcification. Studies on medial calcification in the iliac, breast and femoropopliteal arteries report a relation to older age, diabetes and kidney disease and no consistent relation to hypertension or hyperlipidemia and even an inverse relation with smoking has been reported.(7,10) The different risk factors of the dominant ICAC types correspond well to the risk profiles on intimal and medial calcification found in the literature.(7,10–12) This confirms that our CT score works, at least, for epidemiological studies and that these two types of calcifications exist on a larger scale. A major limitation in vascular calcification

research was that in vivo tests for differentiating intimal and medial calcifications in one vascular bed were lacking. We have now provided a tool for this in this thesis by designing and validation our ICAC score.

The different influence of intimal and medial disease on stroke risk remains to be investigated, although the score has recently been used by others in patients treated with intravenous thrombolytic therapy in acute ischemic stroke.(13) They found dominant medial calcification to be associated with an increase of symptomatic intracerebral haemorrhage and decrease of early response in these patients. This could point at a role for medial calcifications in the pathogenesis of intracerebral haemorrhage.

3. ICAC was found on CT in children below ten years of age, in our study of trauma patients. This was an unexpected finding especially given the absence of CT calcifications in other vascular beds at this young age.(12,14) On a histological level, ICAC found in children is mostly located around the internal elastic lamina.(15) Calcification of the lamina, together with the adjacent arterial media, are often considered to be medial arterial calcification and is found at a similar location in the intracranial carotid artery of older patients.(15,16) At older age ICAC is thought to be multifaceted and caused by a combination of multiple cardiovascular risk factors. It is unlikely that the same risk factors contribute similarly to ICAC in young children as the exposure time to these risk factors is limited.(1) The causes for medial calcification in the carotid arteries of children remains speculative. It may be a repair mechanism for broken elastin fibres with a heritable component.(15,17,18) The clinical implications are at present unclear, although 10% of all strokes occur in patients between 18-50 years of age.(4)

Hippocampal calcification, a new imaging marker for cognitive decline?

Hippocampal calcification has only recently been described on CT images and histopathology. In 2012, Chew et al. were the first to report these calcifications on CT images. They hypothesized that hippocampal calcification was long overlooked on both CT and MRI. On CT it was most likely mistaken for calcification of the adjacent choroid plexus. On MRI, the main imaging modality in neurocognitive research, it is difficult to visualize calcification.(19) Because of the novelty of hippocampal calcification (imaging), its prevalence, etiology, and clinical implications were unknown at the start of our research.

In the study by Chew, hippocampal calcification was found to be present in 21.7% of patients over 50 years of age. This is similar to our memory clinic patients but lower compared the DUST cohort (34%). In the study by Chew, hippocampal

calcification was not reported before 50 years of age whereas it was already present in 8% of patients younger than 40 years of age in the DUST cohort. We confirmed older age to be associated with hippocampal calcifications, and in stroke patients we also identified diabetes mellitus and hyperlipidemia as risk factors. Subsequently, in a memory clinic cohort we confirmed older age, diabetes mellitus, and we found current smoking to be associated. A relation with atherosclerosis appeared likely based on the identified risk factors and early presentation in stroke patients, but the histological basis of hippocampal calcification on CT remained to be confirmed.(20)

In a previous histopathological study, Wegiel et al concluded that hippocampal calcification is associated with loss of neurons and atrophy, most likely through degeneration and occlusion of blood vessels.(20) Interestingly, they deemed hippocampal calcification to be non-atherosclerotic. In a comparative CT-histopathology study we proved that the CT calcifications are indeed of vascular origin and histologically located in the adventitial and medial layers of hippocampal capillaries and arterioles. This does contradict the associations that we found with atherosclerotic risk factors such as hyperlipidaemia and smoking. Apparently at this particular location these classical cardiovascular risk factors were not related to atherosclerotic disease but to calcifications in the medial and adventitial layer.

Previous studies looking into hippocampal function along its longitudinal axis concluded that the hippocampal tail is most sensitive to ischemia and that the dentate gyrus is the most likely place where age related cognitive decline starts.(21–23) In both histology and imaging studies we consistently found that hippocampal calcifications are most often located at these locations (i.e. the dentate gyrus and cornu ammonis 1 area in the hippocampal tail). A relation of hippocampal calcification to cognitive decline and/or dementia is therefore not unlikely.(20,23,24) We tested this hypothesis in a pilot study comparing memory clinic patients to a control group and found that hippocampal calcification was found more frequently present in memory clinic patients. As well, within those patients, the Mini Mental State Exam score of patients with calcifications was lower than in those without. Following the preliminary study, we investigated a larger cohort of elderly memory clinic patients. These patients had undergone extensive cognitive testing using the Cambridge Cognition Examination and Visual Association Test. In this study, no significant relation between calcifications and cognition was found, however, the memory clinic cohort lacked a good control group. Further research is therefore needed to better understand the significance of hippocampal calcification in relation to clinical outcome.

Future Perspective

In this thesis, we have taken several steps into examining intracranial calcification on CT imaging. What are three important future directions?

1) How can the intima-media calcification separation be improved? With the current score, we were able to discern two different risk profiles between intimal and medial calcification in the intracranial carotid artery. Still, optimization is required to improve applicability and diagnostic accuracy. One potential strategy for improvement is increasing the resolution of the CT images. An example of increased resolution is shown in **figure 1**. In preliminary data, the conventional brain CT setting was compared to a different kernel that is used for imaging the mastoid. The increased resolution unveiled subtle calcifications that were not seen on the conventional brain CT scan. Another strategy is multi-parametric analysis, incorporating soft plaque or hemodynamic characteristics to more reliably distinguish calcification type. The properties of CT allow it to excel at imaging calcifications, whereas MRI is optimized for soft tissue imaging, hemodynamic imaging and plaque characterization. Currently, MR imaging of calcification is suboptimal compared to CT, but if calcification imaging would become reliable on MRI it would aid further (longitudinal) research, especially in younger patients.

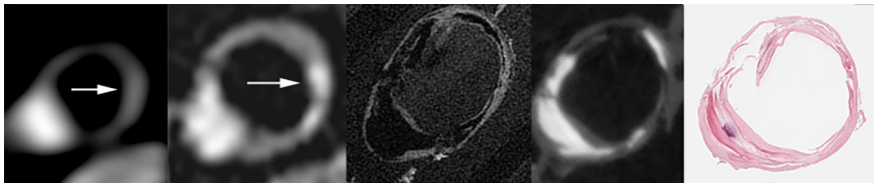

Figure 1 – Imaging of intracranial carotid artery calcification of the same segment with different modalities. From left to right shows conventional brain CT kernel, Mastoid CT kernel, 9.4T MRI and microCT. The corresponding histological slide is shown on the far right. The arrows indicate subtle calcifications which are hardly noticeable on conventional brain CT scans and can be clearly visualised using the higher resolution Mastoid CT kernel.

2) Can intracranial calcification imaging aid therapeutic interventions? Recently, treatment of arterial calcification using bisphosphonates was investigated using CT in a trial with pseudoxanthoma elasticum (PXE) patients, a rare hereditary disease that leads to medial calcification.(25) One of the known factors contributing to medial calcification is a decreased level of inorganic pyrophosphate.(26) The trial showed that treatment with bisphosphonates during one year stopped the

calcification process in the femoropopliteal arteries and lead to fewer ocular events. While treatment options are still in their infancy, we are intrigued by the first results and are interested in the effects on intracranial arterial calcification.

Much of the focus in research on cognitive decline, dementia and Alzheimer's disease is on amyloid beta and tau protein depositions and examination of the anterior hippocampus.(27,28) While a lot of knowledge is gathered in this field, the relation to cognitive decline and possible interventions remain poorly understood. A relation of arterial disease and arterial calcification to cognitive decline and dementia has also been described.(29) For hippocampal calcification, modifiable risk factors (diabetes mellitus and hyperlipidemia) were identified which can be targeted by lifestyle improvements such as (increased) physical activity, lipid lowering drugs and glucose stabilization.(24,30–32) Considering this, we would like to stress that the identified risk factors of ICAC and hippocampal calcification are known to contribute to cardiovascular and cerebrovascular disease in general and a first logical effort would therefore be to focus on preventing or limiting these known cardiovascular risk factors.

3) What is the clinical significance or prognostic value of ICAC in children? To investigate the significance of ICAC in children and young adults can prove difficult. As demonstrated in this thesis, retrospective use of trauma CT scans is a convenient option as no additional exposure to ionizing radiation is required. Case-control studies in populations with early onset stroke can also provide further insight. Lastly, better MR techniques for ICAC imaging would be most helpful for longitudinal prospective studies such as the 'Generation R' or the 'Utrecht Dynamics of the Youth' cohort study.

Main conclusions

In this thesis we showed that intracranial calcification is multifaceted, occurring in multiple arterial layers, and is not synonymous with atherosclerosis. Our findings furthermore suggest that ICAC is the earliest manifestation of arterial calcification in humans, although its relevance in children and young adults remains to be determined. The new insights into hippocampal calcification can hopefully stimulate research to concentrate more on the vascular aspects of neurodegenerative disease and no longer ignore the tail of the hippocampus. Finally, the methods developed in this thesis, using CT and histology, can aid researchers with further investigating medial and intimal calcification in the intracranial carotid artery and hippocampal calcification.

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CHAPTER 10

Summary

Intracranial arterial calcification is a highly prevalent imaging finding that has been related to stroke and dementia. It is reported on CT examination of the brain in the majority of patients over 60 years of age and is most prevalent in the intracranial carotid artery and choroid plexus. The relevance of these calcifications is increasingly recognised but a lot of vital information such as prevalence the general, young population is still lacking. Comparison of CT images with underlying tissue/histology can also provide additional insight in these ectopic calcifications.

Intracranial carotid artery calcification

A lot has been written on intracranial carotid artery calcification (ICAC) on CT in elderly patients and its relation to cerebrovascular disease. Its prevalence in the young and adolescents is currently unknown. To provide an overview of the prevalence of ICAC in the general population from birth to 100 years of age we analysed a large cohort of trauma patients in **Chapter 2**. ICAC was found to be present in 7.4% of patients younger than 10 years of age and prevalence increased with age. Interestingly, no evident difference between genders in terms of prevalence and volume at the different ages was found.

ICAC on CT was always thought to be atherosclerotic, located in the arterial intima, similar to calcification of the coronary arteries. A recent study looking into histology of ICAC found the calcifications not only to be located in the intima but most often in the arterial media. Intimal and medial calcifications are described to have different pathogenesis and clinical implications and could previously only be distinguished through ex vivo histological analysis. Because of these implications we set out to distinguish ICAC in vivo on CT images. In **Chapter 3** we created a calcification score by comparing morphological characteristics of ICAC on CT to the corresponding histopathology in 16 cerebral autopsy patients. The developed score determines the dominant calcification type in the intracranial carotid artery based on thickness, circularity and morphology. The score showed good reproducibility and reasonable ability to differentiate between dominant intimal and medial calcification allowing for further epidemiological studies on this subject.

In **Chapter 4** we applied the ICAC score to the brain CT examinations of 1132 (suspected) stroke patients who were included in the Dutch acute Stroke Trial (DUST). In this study we determined the risk factors of dominant intimal and

dominant medial calcifications and whether there were differences between their respective risk factor profile. Age, pulse pressure and family history were found to be risk factors for both types of calcification. A difference was found which largely corresponded to the known risk factors of intimal and medial calcification. Using multivariable analysis, risk factors for dominant intimal calcification only were smoking and hypertension and for dominant medial calcification diabetes mellitus and previous vascular disease.

Hippocampal calcification

Whereas calcification of the intracranial carotid artery was already examined on x-ray images of the brain in 1965, calcification of the hippocampus was only recently described on CT images. The authors who first reported on hippocampal calcification (HCC) on CT speculated that it was long overlooked due to its resemblance to calcification of the adjacent choroid plexus. The hippocampus is part of the limbic system and plays an important role in the consolidation of memories. Previously, a histological study had concluded that calcification of hippocampal arterioles and arteries could cause atrophy of the surrounding hippocampal tissue. Knowing this we aimed to examine the relation between HCC as seen on CT with the corresponding histopathology in **Chapter 5** to determine whether the hippocampal calcifications that were previously reported were the same form of calcification. All HCC observed on thin slice CT images were confirmed by histological staining to be of vascular origin. Mild HCC in was found in the precapillaries and capillaries of the molecular layer of the DG and CA1. In more severe stages the arteries in the molecular layer of the CA1 and the granular layer of the DG were also affected. Calcifications were most often found in the hippocampal tail and in a more severe stage they also became visible in the hippocampal body and sometimes in the hippocampal head.

Hippocampal atrophy, similar to what was found on histology in HCC, is related to cognitive decline in diseases such as Alzheimer's Disease. In **Chapter 6** we tested whether HCC was related to cognition in a pilot study which included brain CT examinations of 67 patients from a memory clinic that were matched by age and gender to a control group. In this study we found significantly more HCC in patients with cognitive decline as compared to controls. Also, the Mini Mental State Exam score, a measure for global cognitive performance, was significantly lower in those with HCC.

In **Chapter 7** we determined the prevalence and investigated the vascular risk factors of hippocampal calcification in patients with (suspected) stroke in the aforementioned DUST cohort. Hippocampal calcification was present in 381 (34%) patients. We found that HCC prevalence increased with age from 8% below 40 to 45% at 80 years and older. In multivariable logistic regression analysis, age per decile, diabetes mellitus, and hyperlipidemia were significantly associated with HCC which suggests a possible atherosclerotic origin.

Finally, in **Chapter 8** the results from the previous studies on HCC, risk factors, and cognition were tested in a cohort of 1991 patients who visited a memory clinic. During the visit all patients underwent a standard diagnostic work-up including the Cambridge Cognitive Examination, which includes the Mini Mental State Examination, and brain CT scan. We found that increasing age, diabetes mellitus and smoking were associated with an increased risk of hippocampal calcifications. Greater degree of hippocampal calcifications was however not associated with lower cognitive function in patients with memory complaints.

ADDENDA

Dutch summary

Verkalkingen in het eerste deel van de halsslagaders komen veel voor, met name in mensen ouder dan 60 jaar. Deze verkalkingen zijn een voorspeller voor het krijgen van een beroerte. Wat minder goed bekend is, is dat het laatste deel van de halsslagader, welke zich in het hoofd bevindt, ook kan verkalken. Dit deel wordt door zijn vele bochten en gelijkenis met een zwanenhals ook wel de 'sifon' genoemd. Wij denken dat de verkalkingen in de sifon minstens zo belangrijk, zo niet belangrijker zijn dan de verkalkingen in de slagader in de hals. Met computer tomografie (CT) scans van de hersenen zijn deze verkalkingen goed te onderzoeken en studies suggereren dat ze een rol spelen bij het ontstaan van beroerte en dementie. Recent onderzoek heeft ook aangetoond dat verkalkingen in andere slagaders in het hoofd zoals die in de hippocampus, een klein gebied in de hersenen die een belangrijk functie heeft bij het geheugen, ook goed gezien kunnen worden op een CT-scan. Mijn promotie richtte zich op verkalkte slagaders in het hoofd en had als doel om meer over deze verkalkingen te weten te komen.

Verkalking van de sifon

Een eerste belangrijke stap om deze verkalkingen goed te begrijpen, was het onderzoek naar de leeftijd waarop deze verkalkingen ontstaan en te bepalen of er een verschil was tussen mannen en vrouwen. In de literatuur was er weinig informatie beschikbaar over de mogelijke aanwezigheid van deze verkalkingen in kinderen en (jong)volwassenen. Daarom hebben we in **Hoofdstuk 2** onderzoek gedaan naar het voorkomen van verkalkingen van sifon in alle leeftijdscategorieën vanaf de geboorte tot 100 jaar oud. Deze groep bestond uit personen die een CT-scan van het hoofd kregen tijdens een bezoek aan de spoedeisende hulp. Uit dit onderzoek bleek dat verkalking van sifon al in 7.4% van kinderen jonger dan 10 jaar oud te zien is en het met oudere leeftijd steeds vaker voorkomt. In patiënten ouder dan 80 jaar komt het zelfs in meer dan 95% van de patiënten voor. Hierbij bleek er geen verschil te zijn tussen mannen en vrouwen in hoe vaak, en in welke mate de verkalkingen voorkomen. Een belangrijke vraag die na dit onderzoek nog beantwoord moet worden is of de verkalkingen die we bij deze jonge kinderen zien hetzelfde zijn als bij oudere patiënten en dus mogelijk gerelateerd is aan het op vroegere leeftijd krijgen van een beroerte.

Een veel voorkomende aanname over verkalking van de sifon is dat het een gevolg (eindstadium) is van slagadervervetting. Het proces van slagadervervetting speelt zich af in de binnenste laag van de slagader en is vergelijkbaar met slagadervervetting (en de daarop volgende verkalking) in de kransslagaders van het hart. Wij hebben

onderzocht of deze aanname feitelijk ook klopte en dat bleek niet zo te zijn. Eerst hebben we onder de microscoop laten zien dat de meeste verkalkingen op deze plek in de middelste (spier) laag van de slagader voorkomt waar geen vetophopingen zijn. Verkalkingen in vetophoping komen minder vaak voor op deze plek van het lichaam. Dit is een belangrijke bevinding omdat verkalkingen in de verschillende lagen een andere oorzaak en ook andere klinische gevolgen kunnen hebben. De volgende uitdaging was, kunnen we de twee soorten van slagaderverkalking (dus in de binnenste en in de middelste laag van de slagaderwand) ook herkennen en van elkaar onderscheiden op CT-scans. In **Hoofdstuk 3** hebben we een visuele score ontwikkeld die op CT-beelden onderscheid kan maken tussen de twee typen verkalkingen. De score is gemaakt door het vergelijken van verkalkingen op CT-beelden met het kijken naar deze verkalkingen onder de microscoop. In de eenvoudig te leren score, kijken we naar de dikte, circulariteit (hoe rond het is) en morfologie (langgerekt of kleine brokjes) van de verkalking.

Met deze nieuwe methode in handen hebben we in **Hoofdstuk 4** de score toegepast op de CT-beelden van 1132 patiënten die deel uit maakten van de Dutch acute Stroke Trial (DUST), ofwel de Nederlandse studie naar acute beroertes. In deze studie keken we naar de risicofactoren van, en mogelijk verschillen tussen, verkalkingen in de binnenste of middelste laag van de slagader. Risicofactoren voor beide typen verkalking waren 'hoge leeftijd', 'hoge bloeddruk' en 'familiaire belasting'. Met statistische analyse vonden we dat 'roken' en 'hoge bloeddruk' risicofactoren waren voor alleen verkalking van de binnenste laag en 'suikerziekte' en 'eerdere vaatziekte' alleen voor verkalking van de middelste laag. Deze bevindingen ondersteunen mede het idee dat beide typen verkalkingen een andere oorsprong hebben en kunnen worden onderscheiden in patiënten met behulp van CT.

Verkalking van de hippocampus

De hippocampus is een gebied in het limbische systeem van de hersenen en speelt een belangrijke rol bij het geheugen. Afwijkingen in de hippocampus worden frequent gezien bij patiënten met dementie en letsel aan de hippocampus kan leiden tot geheugenverlies. Toen ik aan mijn onderzoek begon waren er in de wereldliteratuur twee publicaties te vinden over verkalking van de hippocampus. In **Hoofdstuk 5** hebben we onderzocht waar de hippocampus verkalkingen, zoals die op CT-scans te zien zijn, precies zitten. Om dit te onderzoeken hebben we zeven hersenen gescand met behulp van CT en vervolgens het onderliggende

weefsel bekeken onder de microscoop. Alle verkalkingen die op CT werden gezien in de hippocampus bleken in de grote en kleine bloedvaten van de hippocampus te zitten. Ze werden het vaakst gezien in de achterzijde van de hippocampus en bij uitgebreidere verkalking ook in het middelste, en uiteindelijk voorste segment. Wij denken dat de verkalkte bloedvaten een oorzaak kunnen zijn voor het krimpen van de hippocampus bij patiënten met geheugenproblemen en dementie.

Als volgende stap hebben we In **Hoofdstuk 6** onderzocht of er een relatie is tussen verkalkingen van de hippocampus en geheugenproblematiek. In deze studie, waarin we 67 patiënten van een geheugenkliniek vergeleken met een controlegroep zagen we meer verkalkingen in de hippocampus in de groep van patiënten met dementie en afname van geheugen. De patiënten met verkalkingen van de hippocampus scoorden ook lager op een geheugentest. De resultaten van deze studie geven een eerste inzicht in de relatie tussen verkalkingen van de hippocampus en het geheugen, al zullen deze resultaten eerst verder onderzocht moeten worden.

Door de beperkte literatuur over hippocampus verkalkingen was er nog geen informatie beschikbaar over de risicofactoren. Het vaststellen van deze risicofactoren kan inzicht geven in het ontstaan van de verkalkingen en mogelijk ook preventie en behandeling. In **Hoofdstuk 7** hebben we daarom gekeken naar de risicofactoren van hippocampus verkalking en hoe vaak deze verkalkingen voorkomen in het eerdergenoemde DUST-cohort. Hippocampus verkalking werd gezien in 281 (34%) van alle patiënten. Bij patiënten jonger dan 40 jaar werd hippocampus verkalking al gezien in 8% van alle patiënten en het nam toe tot 45% bij patiënten van 80 jaar en ouder. In de statistische analyse waren 'oudere leeftijd', 'suikerziekte' en 'verhoogd vetgehalte in het bloed' geassocieerd met hippocampus verkalking. Dit pleit voor een relatie met slagadervervetting.

Afsluitend hebben we in **Hoofdstuk 8** de resultaten van onze voorgaande studies over hippocampus verkalking, in relatie tot risicofactoren geheugenproblematiek, getest in een cohort van 1991 patiënten van een geheugen kliniek. Alle patiënten hadden tijdens hun bezoek een uitgebreide geheugen- en cognitietest ondergaan en een CT-scan van het hoofd gehad. In deze studie vonden we een relatie tussen hippocampus verkalking en 'oudere leeftijd', 'suikerziekte' en 'roken'. De ernst van hippocampus verkalkingen was niet gerelateerd aan verminderd geheugen. Op basis van de resultaten van deze studie kunnen we dus niet concluderen dat hippocampus verkalking gerelateerd is aan een verminderd geheugen hoewel dit

mogelijk verklaard kan worden door een aantal beperkingen van de studie. Ons' inziens zijn er meer studies nodig met een 'gezonde' controlegroep om tot een uiteindelijke conclusie te komen.

Samenvattend, in dit proefschrift hebben wij het bewijs geleverd voor wat CT precies afbeeldt in de sifon en in de hippocampus en een nieuwe methode ontwikkeld om verschillende soorten kalk te onderscheiden. Met deze methoden hebben we de risicofactoren beter kunnen onderzoeken met uiteindelijk als doel een betere behandelstrategie te vinden voor beroerte en dementie.

ADDENDA

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ADDENDA

Curriculum Vitae

Remko Kockelkoren was born on October 30th, 1986 in Zwolle where he also grew up. He completed high school at the Thorbecke Scholen Gemeenschap in 2005 after which he started a bachelor Movement Sciences at the University of Groningen. In 2006 he switched to study Medicine at the same University. During the final year of his master he participated in the medical frontier program of the Osaka University Graduate School of Medicine in Japan. After graduating in January 2014 he started as a PhD student at the Radiology Department of the University Medical Center Utrecht. His research focussed on imaging of intracranial arterial calcification under supervision of professor Willem Mali, professor Pim de Jong and dr. Jill De Vis. During his PhD he published multiple peer reviewed articles, presented his results at the ESR and RSNA, and was a visiting researcher at the Royal Infirmary Edinburgh. During his PhD he also completed a postgraduate Master's program in Clinical Epidemiology. In May of 2017 he started working at the emergency department of the Deventer Ziekenhuis, during which he finished writing this thesis. He is looking forward to starting his training to become a General Practitioner in September 2018 in Zwolle.