Microvascular brain raspberries: A clinicopathological study

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Microvascular brain raspberries: A clinicopathological study

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Photo by Kennet Ruona

HENRIC EK OLOFSSON graduated from Lund University in 2018. He is currently a member of the Division of Pathology led by Professor Elisabet Englund, in parallel with his residency training at Clinical Genetics, Pathology, and Molecular Diagnostics in Lund. The focus of Henric's thesis is a microvascular formation of the human brain encountered during postmortem neuropathological examinations, termed 'raspberry' due to its characteristic appearance when viewed under a brightfield microscope. The abundance of raspberries varies considerably among individuals, but little is known about the characteristics of this variation. In his thesis, Henric examines the clinicopathological context in which raspberries may be more abundant, with the goal of directing future research on the pathogenesis and consequences of this microvascular entity.

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Microvascular brain raspberries: A clinicopathological study

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract:

A raspberry is a microvascular formation of the human brain that can be encountered during postmortem neuropathological examinations. Our histopathological definition of a raspberry connotes a minimum of three vascular lumen surrounded by a common perivascular space and sectioned transversally. Prior to the start of this project, our impression was that the number of raspberries could vary considerably among individuals, but little was known about the characteristics and significance of this variation. As such, the aim of this thesis was to examine the distribution of raspberries in archival brain tissue to specify the clinicopathological context wherein raspberries occur, in an attempt to direct the focus of future research. A hypothesis that guided our study designs was that raspberries form in a setting of chronic or recurrent hypoperfusion.

This was a retrospective study based on archival brain tissue originally sampled as part of postmortem neuropathological diagnostics. To compare the raspberry density between different diagnoses, the raspberries were quantified in tissue sections from the cerebral cortex – mainly in the frontal lobe, since raspberries had been frequently encountered in this region. After retrieval of recorded clinical data and autopsy findings, we examined whether the raspberry density varied in relation to age, large vessel disease, small vessel disease, vascular brain injury, vascular dementia, neurodegenerative disease, and clinical data including hypertension, diabetes, and other diagnoses that have been associated with reduced cerebral blood flow.

The raspberry density increased with advancing age independently of potential confounding factors. Our findings further indicated an increased raspberry density in patients with cerebral atherosclerosis, hypertension subgrouped for organ damage (exploratory), and vascular dementia. Regarding neurodegenerative diseases, the raspberry density was found to be increased in frontotemporal lobar degeneration, but not in Alzheimer's disease or Lewy body disease. In the forebrain, raspberries were frequently encountered in the cortex and deep grey matter, but only rarely in the white matter.

As such, raspberries are a partially age-related phenomenon that becomes more abundant in a setting of high vascular disease burden. The raspberry density is selectively increased in frontotemporal lobar degeneration compared to Alzheimer's disease and Lewy body disease. These findings narrow down the clinicopathological characteristics of raspberries. This knowledge can be applied to direct the focus of future research on the pathogenesis and consequences of raspberries, wherein further applicable methods include transcriptomics, imaging, and animal models.

Key words: cerebrovascular disease, cerebral small vessel disease, vascular dementia, neurodegenerative disease, frontotemporal lobar degeneration, hypertension

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It is not down on any map; true places never are.

Herman Melville

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Populärvetenskaplig sammanfattning

Det här projektet har undersökt en kärlförändring i hjärnan som kallas ‘hallon’, vilket är en referens till dess speciella utseende i mikroskop. Vi började lägga märke till hallon i samband med mikroskopisk undersökning av hjärnvävnad som tillvaratagits vid kliniska obduktioner utförda för att diagnostisera hjärnsjukdomar. I mikroskopet ser ett hallon ut som flera tvärsnittade blodkärl som ligger tillsammans i ett knippe, inneslutna i ett gemensamt rum.

Vi noterade att dessa hallon var ett relativt vanligt fynd, men att mängden varierade betydligt mellan olika personer, och även mellan olika delar av hjärnan. Vi visste dock inte något om vad den här variationen representerade. Kärlsjukdom i hjärnan ligger bakom bland annat stroke och vissa former av demens och trots att mycket forskning bedrivits på dessa tillstånd är mycket fortfarande okänt. Därför är det viktigt att utreda kärlavvikelser i hjärnan och fastställa deras betydelse.

För att åstadkomma detta, började vi kartlägga hallontätheten vid olika sjukdomstillstånd på ett systematiskt sätt. Vi räknade antalet hallon i hjärnvävnadsbitar, framför allt från hjärnbarken (cortex) i frontalloberna, och korrelerade detta med andra sjukdomstillstånd i hjärnan, samt med andra organfynd och kliniska sjukdomar patienten lidit av. Vi undersökte även i mer detalj om hallontätheten varierade mellan olika delar av hjärnan.

I vår första studie studerade vi hallontätheten vid olika demenstillstånd och jämförde den med kontrollfall (fall som var fria från hjärnsjukdom). Vi fann att hallontätheten var förhöjd vid ‘vaskulär demens’, en demensform som uppstår när kärlsjukdom orsakar kronisk blodbrist i hjärnan till följd av blodproppar och andra kärlskador. Vi jämförde även hallontätheten mellan olika delar av cortex, och noterade att den var högre i frontalloberna (pannloberna) jämfört med occipitalloberna (nackloberna).

I den andra studien undersökte vi hallonförekomsten i förhållande till kardiovaskulära riskfaktorer och kliniska tillstånd förknippade med avvikande blodtryck eller blodcirkulation. Vi fann en förhöjd hallontäthet hos patienter med åderförfettning (ateroskleros) i hjärnans stora blodkärl.

Syftet med vår tredje studie var att se om resultaten från studie nummer två kunde bekräftas i en större grupp patienter. Liksom i den föregående studien fann vi en högre hallontäthet hos patienter med ateroskleros i hjärnans kärl, men fyndet var inte lika starkt som tidigare. I denna studie kartlade vi vidare hallontätheten i olika delar av hjärnan, och fann att den var relativt hög i cortex och i andra former av grå hjärnsubstans, men att den var låg i vit substans och i lillhjärnan.

Vår fjärde studie fokuserade på förhållandet mellan hallontäthet och ålder. Vi observerade en ökande hallonförekomst med stigande ålder, men fann också ett samband mellan förhöjd hallonförekomst och högt blodtryck. Detta samband gällde

dock endast om man tog hänsyn till allvarlighetsgraden av det höga blodtrycket genom att kartlägga andra organfynd. Våra resultat tydde även på att mängden hallon var något högre hos kvinnor än hos män.

Den femte studien undersökte återigen förekomsten av hallon vid sjukdomar som kan ge demens; de undersökta sjukdomarna även kan även yttra sig som störningar i kroppens motorik. Den här gången kartlade vi hallontätheten vid tillstånden ’frontotemporal lobär degeneration’ (FTLD) och ’Lewykroppssjukdom’. Vi fann att hallonförekomsten var förhöjd vid FTLD, medan den vid Lewykroppssjukdom inte skilde sig från kontrollfall.

För att sammanfatta, har vi funnit att hallon förekommer i ökad utsträckning i samband med stigande ålder, men att förekomsten ökar ytterligare vid vaskulär demens, ateroskleros i hjärnans stora blodkärl, och högt blodtryck med hänsyn till samtidig organskada. Därtill fann vi en ökad hallonförekomet vid FTLD.

Fynden kan ge vägledning i framtida studier som syftar till att fastställa hallonens uppkomstmekanismer och konsekvenser. Bland annat kan man undersöka genuttrycket i hallonrika hjärnregioner, eller undersöka i djurförsök om hallon kan bildas som svar på vissa stimuli. Detta kan förbättra våra kunskaper om kärlsjukdomar i hjärnan. Därtill behövs ytterligare forskning för att förstå orsaken till den förhöjda hallontätheten vid FTLD, ett tillstånd som inte anses förknippat med kärlsjukdom, men där mycket forskning pågår för att fastställa sjukdomens orsak. Vidare studier behövs också för att fastställa hallonens tredimensionella struktur och sätta dem i relation till liknande fynd av andra författare. Det är möjligt att den tredimensionella strukturen av hallon snarast liknar ett tvinnat rep, eller – för att använda en tematiskt mer närliggande liknelse – en hallonrem.

List of papers

Paper I

Ek Olofsson H, Englund E. A cortical microvascular structure in vascular dementia, Alzheimer's disease, frontotemporal lobar degeneration and nondemented controls: A sign of angiogenesis due to brain ischaemia? Neuropathology and Applied Neurobiology. 2019;45(6):557-69.

Paper II

Ek Olofsson H, Haglund M, Englund E. Are cortical microvascular raspberries caused by cerebral hypoperfusion? An exploratory pathological study. Cerebral Circulation - Cognition and Behavior. 2021;2:100026.

Paper III

Ek Olofsson H, Haglund M, Englund E. On the regional distribution of cerebral microvascular 'raspberries' and their association with cerebral atherosclerosis and acute circulatory failure. Cerebral Circulation - Cognition and Behavior. 2023:100157.

Paper IV

Ek Olofsson H*, Österling Delshammar T*, Englund E. Cortical microvascular raspberries and ageing: An independent but not exclusive relationship. Acta Neuropathologica Communications. 2023;11(1):195.

Paper V

Ek Olofsson H, Englund E. Cortical microvascular raspberries are more frequent in frontotemporal lobar degeneration than in Lewy body disease and control cases: A neuropathological study. Manuscript in preparation.

*Equal contribution

Abbreviations

ACA	Anterior cerebral artery
AD	Alzheimer's disease
ADNC	Alzheimer's disease neuropathologic change
CAA	Cerebral amyloid angiopathy
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma
GRN	Progranulin gene
HIF	Hypoxia inducible factor
LBD	Lewy body disease
MAG	Myelin-associated glycoprotein
MCA	Medial cerebral artery
PCA	Posterior cerebral artery
PLP1	Proteolipid protein-1
TDP-43	Transactive response DNA-binding protein 43
UPS	Ubiquitin-proteasome system
VaD	Vascular dementia
VEGF	Vascular endothelial growth factor

Background

Overview

The focus of this project has been the raspberry, a term used to describe the histopathological appearance of a microvascular formation that has been observed in brain tissue sections during clinical neuropathological examinations. The term raspberry stems from the characteristic, histopathological appearance of the formation (Fig. 1–2). Our definition of a raspberry connotes a microvascular formation consisting of three or more adjacent vascular lumen, surrounded by a common perivascular space and sectioned transversally.

At the start of the project, we had identified raspberries in various neuropathological conditions during clinical postmortem examinations, as well as in patients with no other major neuropathological findings. It was apparent that the number of raspberries could vary considerably among individuals, as well as between different anatomic locations within the same individual, but the significance of this finding was unknown.

Being part of a clinical lab with access to archival brain tissue, the most natural approach to examine raspberries was to quantify the occurrence of raspberries in different diagnoses to generate hypotheses on the pathogenesis of this entity. While this approach does not test all hypotheses directly, it is a non-invasive way to direct future experimental or prospective approaches.

However, to discuss the results acquired from these clinicopathological studies, one must provide a background. The first part of this background attempts to overview some mainly physiological aspects of blood vessel formation, anatomy of the cerebral vasculature, and regulation of cerebral blood flow. The later parts of the background focus on cerebral ischaemia and other forms of vascular pathology, in a general context and then in relation to vascular dementia and neurodegenerative disease. The final part of the background describes findings of other authors on vascular entities that overlap with raspberries.

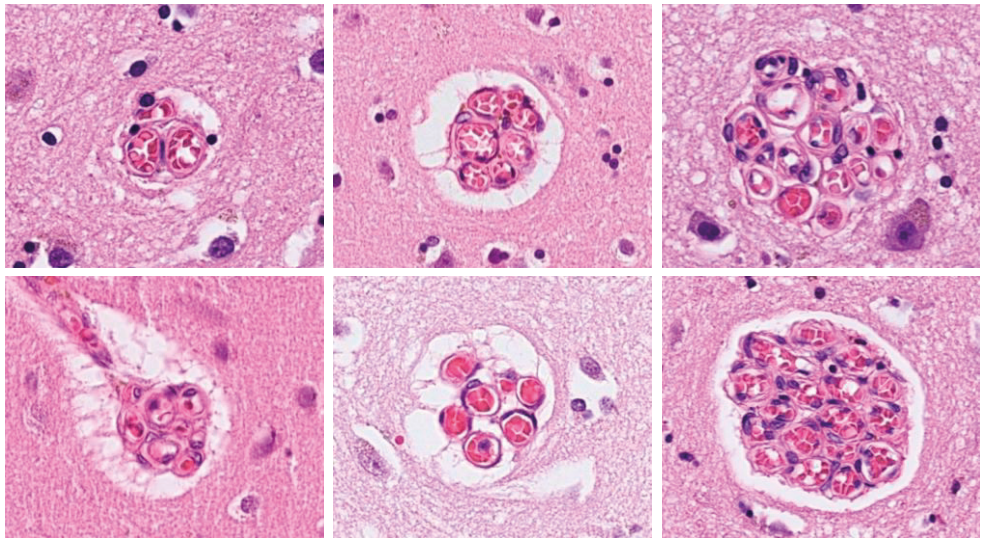

Fig. 1
Examples of cortical microvascular raspberries.

Blood vessel formation

To maintain the environment within a favourable niche that admits cellular life and functionality, a steady supply of nutrients is required, as well as a means to dispose of waste products. Bacteria can rely on simple diffusion to maintain this niche, but in humans, diffusion becomes insufficient early during development, as the growing embryo reaches but a few mm in size [1]. This problem is shared by most multicellular organisms; a transportation system to maintain homeostasis has been in high demand since an early point of evolution [2]. In humans and other vertebrates, the vascular system represents the solution to this challenge, allowing the delivery of oxygen and nutrients by arteries and the disposal of carbon dioxide and other waste products by veins and lymphatics.

Certain aspects of embryonic development can illustrate the formation and organisation of blood vessels. The vascular system begins to form during the third week of embryonic development, as a primitive capillary network is laid down by endothelial precursor cells derived from the mesenchymal germ layer – a process termed ‘vasculogenesis’ [1]. This network interconnects with the developing heart as it starts beating during the fourth week of pregnancy, at a point when the embryo reaches about 4 mm in size [1]. Around this time, the neural tube has separated from the rest of the ectodermal germ layer and has already acquired a rudimentary segmentation in the form of primary vesicles (forebrain, midbrain, and hindbrain)

[3]. The vascularisation of the brain is initiated as a vasculogenesis-derived capillary network (the perineural vascular plexus) forms around the neural tube [3]. The vascularisation of the neural parenchyma is then achieved mainly through 'sprouting angiogenesis' – that is, the formation of new blood vessels from pre-existing ones [4].

Capillary sprouts are led by endothelial tip cells equipped with filopodia that can extend or detract accordingly in response to proangiogenic stimuli [5]. The tip cells are followed by endothelial stalk cells that proliferate, form a lumen, and synthesise a new basement membrane around the developing capillary, along which migrating pericytes join and stabilise the vessel [4]. When tip cells from separate sprouts meet, the sprouts fuse, thus creating an anastomosis between two vascular beds. During embryonal development, capillary sprouts penetrate the neural parenchyma and grow radially towards the ventricles, guided by chemoattractant signals from neuronal progenitor cells [6].

In the embryo, the initial formation of the vascular system is achieved through a series of genetically predetermined steps [7]. The continued development, including the expansion of the capillary network, the regression of non-perfused vessels, and the differentiation into arteries and veins, is believed to take place in interaction with physiological stimuli [7], mainly hypoxia and flow-mediated effects on the endothelium [8, 9]. Going from a homogenous capillary network to a hierarchical and organ-specific vascular system requires the combined influence of multiple signalling molecules. A central molecule is vascular endothelial growth factor A (VEGF), which induces and guides sprouting angiogenesis, while also interacting with other angiogenic and vasculogenic signalling pathways [5, 10]. Due to steps involving degradation of the basement membrane and induction of vascular permeability, VEGF has a destabilising effect on the vasculature, and is tempered by other factors, such as angiopoietin 1 and 2. Angiopoietin 1 promotes maturation and stabilisation of the vasculature, while angiopoietin 2 can induce either angiogenesis – in conjunction with VEGF – or vascular regression, when VEGF is absent [11].

VEGF further interacts with neurogenic signalling pathways and as such, has a role in the development of the brain as well as the vasculature [12]. As such, the development of the vascular system and the nervous system are partially co-dependent and converge in the formation of a 'neurovascular unit', a term connoting the structural and functional interconnection between neurons, glia, and blood vessels [13].

Embryonic development represents a physiological form of blood vessel formation that continues during foetal and postnatal development [10, 14]. In the adult, the vasculature has acquired a stable and quiescent phenotype, in contrast to the highly dynamic and constantly remodelling vasculature of the developing brain [10]. However, the adult vasculature maintains the capacity for angiogenesis (and

potentially vasculogenesis), which can be reinitiated in response to various stimuli through increased signalling of VEGF and other molecules [15]. Similar to developmental stages, hypoxia is an important stimulus for adult angiogenesis by increasing the activity of hypoxia inducible factor 1 (HIF-1), which promotes the transcription of genes encoding VEGF and many other proteins [8]. Moreover, adult angiogenesis is often influenced by co-occurring inflammation, as many inflammatory mediators have angiogenic effects [16]. Ultimately, the switch from a stable, quiescent vasculature to a destabilised and angiogenic one requires an interplay between proangiogenic, antiangiogenic, and stabilising factors.

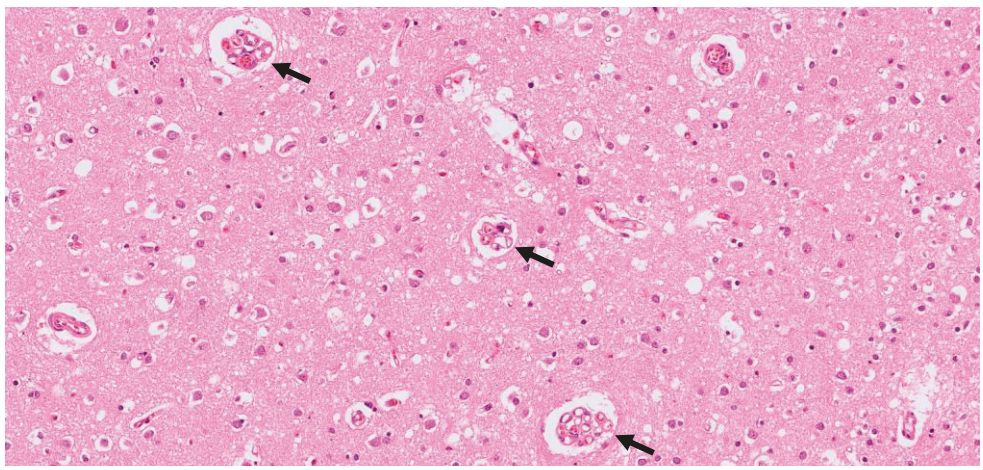

Fig. 2
Examples of cortical microvascular raspberries.

Vascular anatomy of the brain

The result of neuronal and vascular development is a richly vascularised organ with regional variations in vascular anatomy that has some functional implications. This section summarises the brain's vascular anatomy, with some emphasis on the arteriolar architecture.

The brain is supplied by the internal carotid arteries anteriorly and the vertebral arteries posteriorly [17]. After entering the cranium and penetrating the dura mater at the base of the brain, the internal carotid arteries give rise to the anterior cerebral arteries (ACAs) and the medial cerebral arteries (MCAs) [17]. The vertebral arteries, in turn, fuse into the basilar artery that terminates in the posterior cerebral arteries (PCAs) [17]. Together with anterior and posterior communicating arteries, the cerebral arteries form the anastomosing circle of Willis at the base of the brain [17]. The cerebral arteries and their branches are located in the subarachnoid space;

the vessels themselves are further surrounded by pial sheaths that are continuous with the pia mater [18].

From their location at the base of the brain, the arteries provide penetrating arteries that supply the deep grey matter (basal ganglia, diencephalon) and the capsula interna [19]. These arteries enter the parenchyma at right-angles [19]. The penetrants are surrounded by a double sheath of pia mater, which may constitute the anatomical basis of widened perivascular spaces around these vessels – a frequent feature of small vessel disease (see later sections) [20]. After providing these penetrating arteries, the cerebral arteries extend along the cerebral hemispheres. Throughout their remaining trajectory, the cerebral arteries give off multiple branches, creating an anastomosing network of pial arteries and arterioles that supply the cerebral cortex and hemispheric white matter [21].

The borders between the territories supplied by the ACAs, MCAs, and PCAs are referred to as watershed areas or simply border zones. As the blood supply to these areas is the most distal from the heart, these regions are considered the most vulnerable to hypoperfusion during haemodynamic failure [22]. The cortical border zones within the pial network can be referred to as 'external', while the 'internal' border zones are found between the arteries that penetrate from the base of the brain on the one hand, and the medullary arterioles (see next section) that originate from the pial network on the other [23]. Borders between medullary arterioles originating from different cerebral arteries also represent internal border zones [22]. The territories within the external border zones encompass the cortex and subjacent white matter; with some interindividual variability [24], they are located slightly parasagittal from the midline between the ACA and the MCA anteriorly, and between all three cerebral arteries posteriorly [22]. The internal border zones within the white matter correspond to the centrum semiovale and corona radiata [22].

The pial network give off arterioles that penetrate the parenchyma perpendicular to the surface [21]. Unlike the penetrants from the base of the brain, these vessels are covered by a single sheath of pia mater; this may be the reason why cortical arterioles seldom exhibit widened perivascular spaces [18, 25].

The penetrating arterioles have their own 'private' basement membrane which is separate from that of the astrocytes constituting the glia limitans [18]. The interposing space between the basement membranes, as well as the vascular wall itself, may house occasional monocytes, macrophages, and fibroblasts [26]. The arterioles further possess a continuous layer of smooth muscle cells and an incomplete internal elastic lamina [18].

At the level of capillaries and small/precapillary arterioles, the vascular and glial basement membranes fuse [27]. The arterioles terminate in capillaries, which are covered by pericytes in a discontinuous manner, and share their basement membrane with astrocytic end feet [27].

The cerebral arteries and the pial network which they supply are extrinsically innervated by sympathetic, parasympathetic, and sensory nerve fibres along the tunica adventitia [28, 29]. The intraparenchymal arterioles (post fusion of the basal lamina) are intrinsically innervated by projections from cholinergic (nucleus basalis of Meynert), adrenergic (locus coeruleus), and serotonergic (raphe nucleus) projections, in addition to the neuronal projections originating from these nuclei [29, 30].

The intracerebral arterioles can be classified depending on their lengths as cortical (terminating in and supplying the cortex) or transcortical (terminating in the white matter) [31]. The transcortical arterioles are of two types: subcortical – providing some branches to the cortex in addition to supplying superficial white matter – or medullary, continuing through the cortex towards the deep white matter without branching [21].

The cortical arterioles have been subclassified in different ways by different authors [21, 31, 32] (Fig. 3–4). In summary, the arterioles can terminate and branch at three main levels. The superficial level corresponds to cortical layers I–II, where the branches typically take off at 90-degree angles from the mother vessel; this is the least common pattern. The intermediate and deep levels correspond approximately to cortical layers III–IV and V–VI, respectively. Here, the vessels branch at acute angles from the mother vessel, resulting in an oblique trajectory initially. These branches can ramify further, and some of them will have a recurrent course (making a U-turn back towards the cortical surface). The visual appearance of this pattern of branching has been compared to fountains or candelabras [32, 33].

The cerebral capillary network rising from the intraparenchymal arterioles is estimated to contain half of the cerebral blood volume at any given time and run as much as 400 miles throughout the brain [10, 27]. The endothelium, pericytes, and astrocytic end feet constitute the blood brain barrier, a physical and metabolic gatekeeper that mediates selective transportation into (or out from) the brain, as well as metabolism of unwanted molecules [34]. In the cortex, three capillary layers have been recognised, roughly corresponding to the arteriolar levels of termination and branching [21, 31]. A fourth layer – the subpial zone – exists but is considered practically devoid of capillaries [21, 31]. The capillary network centred around cortical layer IV is indicated to have the highest capillary density [21, 31]. The capillary density of grey matter is considerably higher than that of the white matter, although results regarding the precise ratio vary – a recent estimate indicated that grey matter capillaries may be up to 6 times as numerous as those in the white matter [35].

The brain drain (of blood) is achieved by deep and superficial venules and veins; these vessels are sparser than the arterioles, generally of a larger diameter, and cover larger volumes of brain parenchyma [21]. The veins transit from the parenchyma to the cerebral convexities and drain into venous sinuses – that is, endothelium-lined

channels formed by focal separations of the two layers of the dura mater [19]. The sinuses empty mainly into the internal jugular veins which carry the blood back to the heart [19].

Other pathways take care of the drainage of interstitial and cerebrospinal fluid; these pathways have been implicated as important for clearance of waste products such as amyloid beta. Possible exit routes for interstitial fluid include retrograde, vasomotion-driven drainage within the walls of penetrating arterioles and pial arteries (intramural periarterial drainage pathway) [27], as well as pulsation-driven flow of cerebrospinal fluid along the paravascular arteriolar space, followed by aquaporin 4-enabled flow into the brain parenchyma and onwards to the paravenular side, followed by exit along the walls of veins, or into the cerebrospinal fluid (glymphatic system) [36, 37]. The main route for exit of cerebrospinal fluid connotes arachnoid granulations into venous sinuses [19].

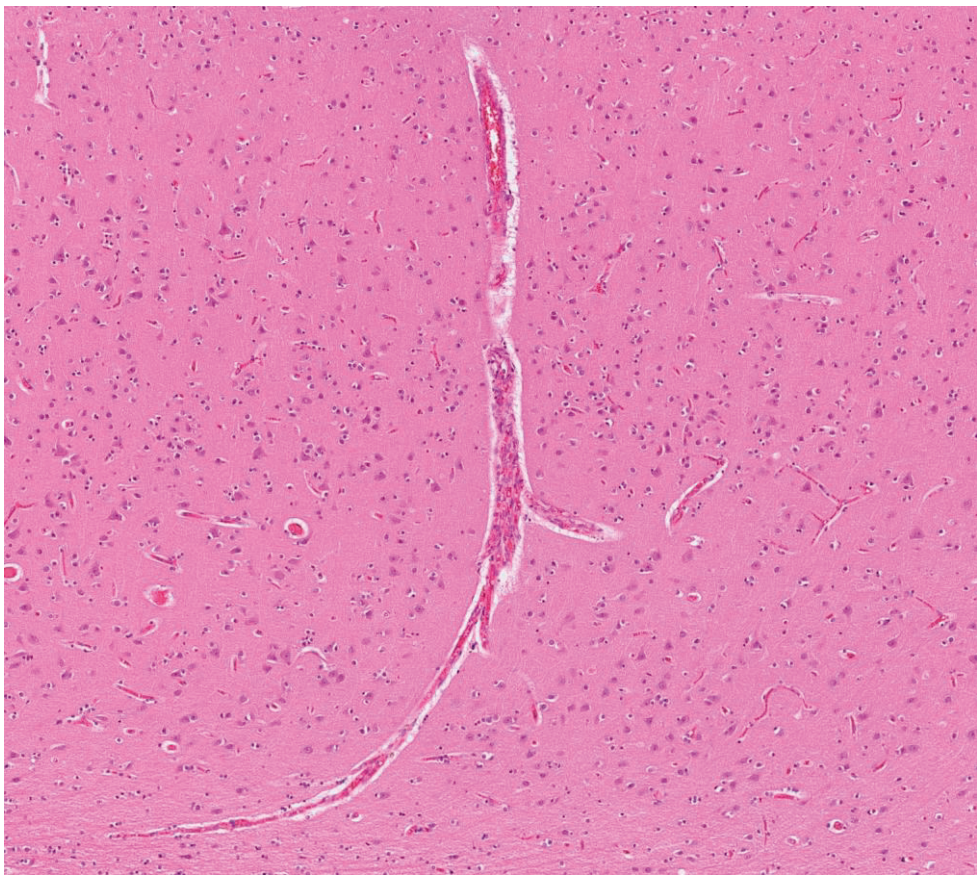

Fig. 3
Overview of cortical arteriolar branching.

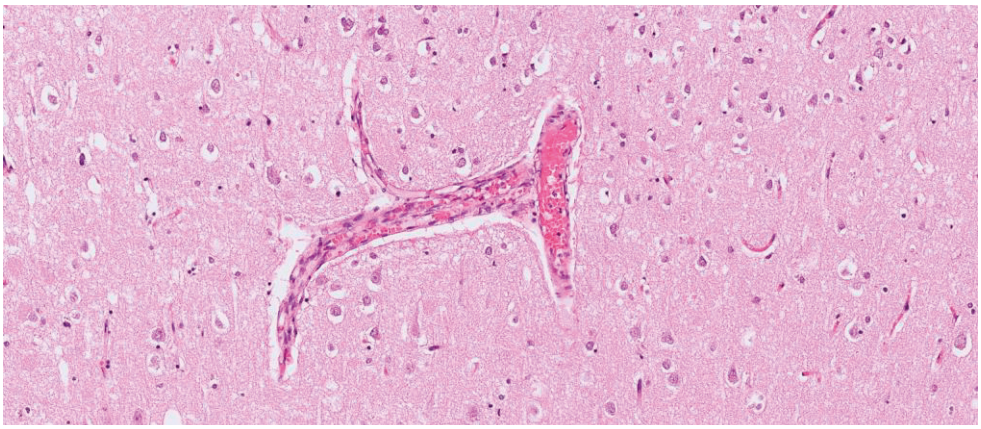

Fig. 4
Cortical arteriolar branching with a minor recurrent branch.

Cerebral blood flow and its regulation

While I cannot provide a reference, the energy consumed while writing these words are not primarily required for my fingers, but my brain. The brain is a demanding customer; despite that it constitutes merely 2% of the body weight, its consumption of 15% of cardiac output and 20% of the total oxygen supply makes it the most metabolically active organ of the body [38]. Normal blood flow to the brain has been estimated to 50 ml/100 g tissue per minute [39] but is unevenly distributed and significantly higher in grey than white matter [40]. The internal carotid arteries provide approximately 70% of the brain's blood flow; the remaining 30% are provided by the vertebral arteries [41]. Due to its lack of glycogen deposits, the brain is dependent on a constant blood flow to sustain its high and fluctuating needs [42].

To meet the high energy demands, the cerebral vascular network is tailor-made not only in structure but also in its regulatory function. To 'serve' the brain, the vasculature can respond to systemic circulatory changes as well as locally mediated stimuli that exert a regional influence on the perfusion. Cerebral blood flow is defended against blood pressure alterations by the baroreflex and by cerebral autoregulation. In response to a drop in blood pressure, stretch-sensitive baroreceptors in the carotid sinus and aortic arch evoke vasoconstriction in other, non-cerebral parts of the circulation, as well as an increase heart rate [43]. Cerebral autoregulation, in turn, connotes the ability of cerebral arteries and arterioles to alter their diameter in response to changes in blood pressure in order to maintain a constant blood flow [44]. Increased wall tension causes muscular contraction, while reduced tension results in vasodilation; as such, cerebral autoregulation acts as a buffer that minimises the influence of blood pressure on cerebral blood flow [45].

The effector cells of cerebral autoregulation – the vascular smooth muscle cells – have an intrinsic ability to respond to changes in wall tension [46]; the response may be further modulated or assisted by flow-mediated effects on the endothelium, or by extrinsic sympathetic innervation [29, 47]. While the relative contributions are incompletely understood, cerebral autoregulation can be executed at multiple levels of the vascular tree, from large arteries to parenchymal arterioles [47, 48]. The same vessels further have the ability to react to alterations in carbon dioxide via lowered extracellular pH [49].

Regional alterations in cerebral blood flow are orchestrated by the neurovascular unit. Principally, neuronal activation induces a corresponding increase in local blood flow; the regulation is termed neurovascular coupling, and the resulting increase in blood flow can be referred to as functional hyperaemia [13]. Signalling in the opposite direction – wherein neuronal activity is adapted according to alterations in cerebral blood flow ('vasculoneuronal coupling') – has also been described [50].

The signalling of neurovascular coupling can be initiated by the activated neurons directly or by activated astrocytes. Glutamate binding to receptors that mediate neuronal depolarisation is paralleled by local release of vasoactive substances such as nitric oxide and arachidonic acid metabolites upon the blood vessel [13]. The response may be modulated further by consequences of neuronal activity, such as hypoxia, adenosine, and potassium ions [13]. The vasoactive substances impact on smooth muscle cells and lead to vasodilation; pericytes constitute additional effector cells [51]. The chemical signalling may also evoke electrical, endothelium-mediated propagation of the signal to upstream arteriolar segments via gap junctions [52, 53]. Intrinsic vascular and neuronal projections from nucleus basalis of Meynert (cholinergic) and locus coeruleus (adrenergic) can alter vascular tone and may play a role in modulating the extent to which incoming stimuli results in increases of blood flow and neuronal activity [30].

Last but not least, the endothelium itself has an important role in maintaining normal blood flow and homeostasis. The endothelium contributes to modulation of vascular tone in response to altered blood flow (shear stress) or in interaction with vasoactive substances, for example, by conversion of angiotensin I to angiotensin II [54]. Additionally, the endothelium normally expresses an anti-inflammatory, anti-thrombotic, and anti-proliferative phenotype that inhibits leucocyte adhesion, thrombosis, and smooth muscle cell proliferation [54]. Many of these functions are thought to depend on endothelial production of nitric oxide, in turn dependent on normal (laminar) shear stress [54].

In summary, the brain is dependent on cardiac output, baroreflexes, cerebral autoregulation, neurovascular coupling, and healthy endothelium for normal blood flow. Due to high and shifting energy demands and insignificant glycogen deposits, the cardiovascular system needs to deliver the right amount of blood at the right

place and time. The blood brain barrier must protect the integrity of the brain, while removal of waste products is achieved via venous blood flow and drainage of interstitial and cerebrospinal fluid.

Cerebral hypoperfusion in a setting of altered vascular function or altered haemodynamics

Stress on the vasculature comes in many forms, including abnormal blood flow that induces pathological shear stress and wall-tension on the endothelium [55], altered hormonal state such as insulin resistance [56] or increased angiotensin II levels [55, 57], altered nutritional state such as hyperglycaemia [56] or dyslipidaemia [56], and chronic inflammation [58]. Some important mediators of vascular stress include hypertension [55], diabetes mellitus [56], smoking [59], obesity, and age [58, 60]. While not the only mechanism, vascular insults often converge into oxidative stress that reduces the availability of endothelial nitric oxide and evokes a shift in endothelial phenotype, affecting vasomotion (impaired vasodilation), inflammation, haemostasis, permeability, and proliferation [54]. The shift in phenotype may be adequate in an acute context, but in a state of chronic vascular stress, the change in function may turn into dysfunction, and ultimately result in remodelling of the vascular structure [54]. Both the functional and the structural alterations can affect the vascular capacity of normal blood delivery (hypoperfusion), the capacity to maintain the integrity across the vessel wall (blood brain barrier integrity), and normal drainage of interstitial fluids (perivascular or paravascular).

Since a hypothesis of ours connotes that raspberries are formed due to chronic cerebral hypoperfusion, some additional space will be used to cover this phenomenon, starting in this section with hypoperfusion from a perspective of altered vascular function and altered haemodynamics.

To my knowledge, there is no consensus on what to include in the term ‘chronic cerebral hypoperfusion’, but potential candidates are reduced resting blood flow, recurrent temporary reductions in blood flow, and dysfunctional neurovascular coupling with a mismatch between supply and demand [61, 62]. In animal models of chronic hypoperfusion, the hypoperfused state is often induced by bilateral common carotid artery occlusion, which results in structural changes similar to human small vessel disease [63]. In humans, however, the potential causes of chronic hypoperfusion are multifactorial. From a functional perspective, vascular stress that causes endothelial dysfunction may contribute to hypoperfusion due to impaired vasodilation [64]. Conditions such as hypertension, a likely contributor to endothelial dysfunction [55], has been associated with reduced cerebral blood flow [65, 66]. While the overall evidence is considered weak [67], individual studies have

indicated that cerebral blood flow can improve after antihypertensive treatment [68, 69], perhaps due to reversal of functional alterations.

One could also apply a more direct haemodynamic perspective on hypoperfusion, such as in acute global hypoperfusion caused by cardiac arrest with return of spontaneous circulation, or other forms of acute circulatory failure. In these cases, the normal regulatory mechanisms of cerebral blood flow are overrun by extreme deviations from normal blood pressure, leading to global hypoperfusion and ischaemia. In a chronic setting, milder forms of reduced cardiac output and reductions in blood pressure are considered potential contributors to ischaemic injury and cognitive impairment [70]. As such, additional examples of potential contributors to chronic cerebral hypoperfusion include heart failure, arrythmias, and orthostatic hypotension [62, 70].

Heart failure has been associated with reduced cerebral blood flow [71-73], white matter hyperintensities [74, 75], and atrophy of the medial temporal lobe, deep grey matter, and limited neocortical regions [76-78]. Likewise, atrial fibrillation has been associated with reduced cerebral blood flow [79] and brain atrophy, either globally in grey and white matter [80], or regionally, affecting hippocampus [81]. Orthostatic hypotension, in turn, has also been associated with white matter hyperintensities [82], although it is not known to what extent reduced cerebral blood flow due to orthostatic hypotension contributes to these lesions [83, 84].

Further indications that heart failure and arrhythmia can affect cerebral blood flow in a chronic, non-acute context include improved cerebral perfusion after cardiac transplantation [71], and the finding that persistent rather than paroxysmal atrial fibrillation was associated with reduced blood flow [79]. In atrial fibrillation, the observed association with brain atrophy was not affected by anticoagulatory treatment [80].

However, it is not known to what extent the mechanisms behind reduced blood flow in these conditions overlap with other effects – such as chronic inflammation and altered hormonal signalling – which could also occur in a state of chronic heart disease and potentially contribute to endothelial dysfunction [85]. Regardless of the mechanism, these conditions have been associated with reduced cerebral blood flow, and were as such included in some of the papers of this thesis.

Structural alterations of blood vessels

Structural effects on blood vessels differ depending on the vascular calibre and can be divided into large vessel disease and small vessel disease.

Large vessel disease

The typical large vessel disease is atherosclerosis, wherein vascular stress causing endothelial injury and dysfunction is thought to predispose for accumulation of lipoproteins in the intima of the vessel wall [86]. The lipoproteins attract the attention of macrophages that unsuccessfully attempt to scavenge the lipids [86]. The macrophages recruit other cell types to the cite, including migrating smooth muscle cells, which proliferate and synthesise connective tissue; the developed plaque consists of a necrotic core of lipid and cellular debris encapsulated by a fibrous cap [86]. The plaque may result in luminal narrowing; in tandem, hypoperfusion of the underlying muscular tunica media due separation from the intraluminal blood supply may cause atrophy and weakening of the vessel wall [86]. The proportion of necrotic core and fibrous cap determines the stability of the plaque and may decide its fate; more necrotic and less fibrotic plaques are considered more prone to rupture [86].

Atherosclerotic plaques tend to form in the vicinity of arterial bifurcations, where the blood flow is turbulent and causes abnormal shear stress [87]. Regarding the cerebral vasculature, the carotid arteries are commonly affected; atherosclerosis in the cerebral arteries at the base of the brain is also frequently encountered [88]. Age, hypertension, and diabetes mellitus can contribute to progression of atherosclerosis into smaller and more distal arteries, sometimes involving the pial arterial network; this is considered a form of small vessel disease [89].

One consequence of atherosclerosis in the cerebral vasculature is progressive luminal narrowing, which could predispose for watershed infarcts in a scenario where severe stenosis co-occurs with acute circulatory failure, either on a pure haemodynamic basis or in combination with impaired washout of microemboli [23]. An atherosclerotic plaque may also undergo acute change due to bleeding within the plaque, rupture of the cap, or ulceration of the endothelium, with subsequent thrombosis [86]. The resulting thrombi can either occlude the arterial lumen of the artery or emit emboli that travel to more distal parts of the vasculature. By these mechanisms, cerebral atherosclerosis may contribute to large infarcts as well as microinfarcts [90]. Atherosclerosis in the carotid arteries may also impair the stretch-sensitive baroreflex due to increased arterial rigidity, thus increasing the risk of orthostatic hypotension and abnormal blood pressure variability [91]. Imaging studies have further associated cerebral atherosclerosis with reduced cerebral blood flow [92] and white matter hyperintensities [93, 94], although such findings need not infer that the observed changes in blood flow and white matter were caused by the atherosclerosis.

Another alteration of large vessels connotes stiffening of elastic arteries due to replacement of deteriorated elastic fibres by collagen or by calcification; the reduced compliance is manifest as increased arterial pulsatility [95]. Like atherosclerosis, arterial stiffening may impair the baroreflex, and has been independently associated

with orthostatic hypotension [96]. Arterial stiffening is considered an age-related feature but may be accelerated by hypertension and other conditions; arterial stiffening may, in turn, increase the risk of hypertension [55]. Increased pulsatility has been associated with white matter hyperintensities and widened perivascular spaces [97]; one theory is that extended propagation of pulse waves into the microvasculature contributes to the development of small vessel disease [95, 98].

Small vessel disease

Cerebral small vessel disease connotes pathologies that affect the vessels of the pial network and the intraparenchymal vessels [89]. Atherosclerosis can extend into the pial network; intraparenchymal arterioles do, however, often not exhibit lipid accumulation [26]. Rather, they accumulate plasma proteins and extracellular matrix that results in hyaline thickening of the vessel wall [86, 99, 100]. As the disease progresses, the smooth muscle cells of the media degenerate and the lumen becomes increasingly narrow [26]. This type of small vessel disease is referred to as 'arteriolosclerosis' [26]. Arteriolosclerosis frequently occurs in the white matter and deep grey matter [101]; the cortex is usually less affected [101], although arteriolosclerosis can occur in this part of the brain as well [102].

Animal models of induced chronic cerebral hypoperfusion demonstrate small vessel disease similar to arteriolosclerosis, indicating that hypoperfusion could contribute to the development of this vascular pathology [63]. While the risk factors of arteriolosclerosis are likely multiple and heterogeneous [26, 103], two main risk factors are generally considered to be hypertension and age [104, 105].

Potential consequences on brain parenchyma affected by arteriolosclerosis include reduced resting blood flow, impaired neurovascular coupling, compromised integrity of the blood brain barrier, and impaired drainage of interstitial fluid. Imaging correlates of small vessel disease, such as white matter hyperintensities, have been associated with reduced cerebral blood flow [106], but do not indicate causality: Does the reduction in blood flow reflect inadequate blood supply (hypoperfusion), or reduced blood flow secondary to reduced metabolism (neurovascular coupling)? (Or reduced metabolism secondary to reduced blood flow [vasculoneuronal coupling] [50]?) Other results on white matter hyperintensities include impaired cerebrovascular reactivity suggestive of impaired vasodilatory capacity [107], increased oxygen extraction fraction indicative of inadequate perfusion [108], and increased leakage over the blood brain barrier [109].

Neuropathological approaches to identify signs of vascular dysfunction in a context of arteriolosclerosis or other small vessel disease include immunohistochemical labelling and other biochemical analyses. Labelling of molecules suggestive of endothelial activation shows conflicting results; a proinflammatory shift, not necessarily indicative of endothelial dysfunction, may occur some contexts [110],

but is not a universal feature of arteriolosclerosis [111]. The endothelium may also acquire a protective phenotype that mediates anticoagulation [111]. Regarding hypoperfusion, a reduced quotient of myelin-associated glycoprotein (MAG) and proteolipid protein-1 (PLP1) has been associated with the severity of arteriolosclerosis in white matter disease [112]. Since MAG is more susceptible to ischaemia than PLP1, a reduced MAG/PLP1 quotient has been applied as a biochemical marker of hypoperfusion; due to their slow turnover, a reduced quotient is further indicative of chronicity [113]. Regarding the blood brain barrier, plasma proteins within the brain parenchyma – suggestive of leakage – has been observed in some contexts [114]. Widened perivascular spaces – a feature that may accompany arteriolosclerosis in white and deep grey matter – has been proposed a potential correlate of altered perivascular drainage of interstitial fluid [25].

Impaired drainage of interstitial fluid leads us to another important form of small vessel disease, namely cerebral amyloid angiopathy (CAA). Functional or structural alterations in the vessel wall that impairs vasomotion and interstitial fluid drainage has been proposed to contribute to the deposition of amyloid withing vessel walls [25, 27]. CAA occurs in 90% of patients with Alzheimer's disease but can also occur independently of this pathology [115]. It most commonly involves pial and intraparenchymal cortical arterioles, but may also affect capillaries and venules [101, 115]. Affected blood vessels may exhibit degeneration of smooth muscle cells and progressive weakening of the vessel wall [101]. CAA can exert deleterious influence as a small vessel disease, wherein potential consequences include microinfarcts [116, 117], capillary occlusions [118], white matter disease [112, 119], and possibly further impairment of interstitial fluid drainage [25, 120], in addition to lobar haemorrhages and microhaemorrhages [101].

Another less studied small vessel alteration of potential pathological relevance is arteriolar tortuosity [121] (Fig. 5). From a small vessel perspective, tortuous arteries and arterioles have been encountered mainly in the white matter and deep grey matter [122]. They can co-occur with arteriolosclerosis in the same vessel [26]. Their abnormal trajectory may predispose for with altered blood flow and hypoperfusion depending on its severity [122, 123]. Neuropathologically, their clinical significance is uncertain. Individual studies have associated them with clinical hypertension [124] and lacunes in the basal ganglia [125]; a tendency towards an association with white matter disease has also been reported [126]. In contrast, a recent imaging study found a negative association with hypertension [127]. However, an association with hypertension would be in line with the clinicopathological characteristics of arterial and arteriolar tortuosity occurring in other part of the body [122].

Some additional small vessel alterations, including altered vascular density, increased basement membrane thickening, capillary string vessels, and morphological signs of altered capillary diameters will be mentioned in their respective contexts in the following sections.

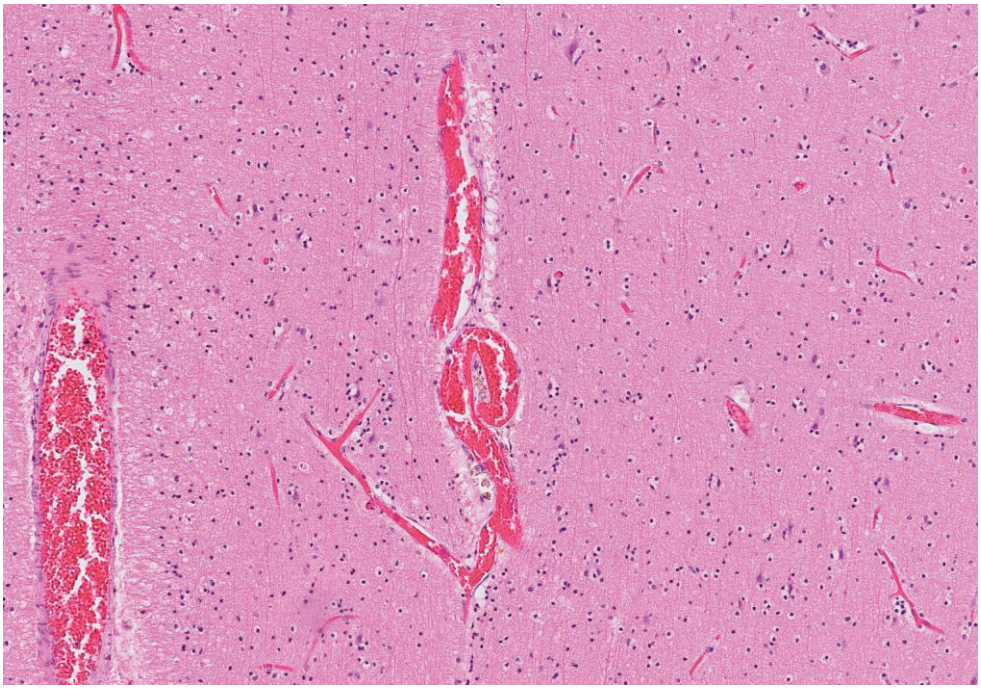

Fig. 5.
Overview of tortuosity/loop formation at the transition from cortex to white matter.

Parenchymal reactions to acute ischaemia, and neuropathological correlates

When a vessel is occluded by stenosis, thrombosis, or embolism, acute focal ischaemia ensues, and ATP-depleted neurons are no longer able to maintain ion gradients over the cell membrane. The net flux of ions results in depolarisation of the neuron, as well as cellular oedema caused by osmotic water influx [128]. Net influx of calcium ions activates multiple catabolic enzymes – potentially ending in necrosis or related death mechanisms – as well as intrinsic apoptotic pathways [129, 130]. The calcium influx also evokes glutamate release, which can trigger depolarisations in other neurons; excessive depolarisations in the hypoperfused penumbra surrounding the infarct core may worsen the energy deficit and contribute to neuronal death [131]. In the end, the neuronal fate of apoptosis, necrosis, or survival is decided by the combined influence of multiple parameters, such as the duration and severity of the ischaemia [132], the impact of recruited inflammatory cells [129], and the functionality and plasticity of the vasculature [133].

Neuropathologically, an acute infarct is characterised by necrosis, which becomes visible as pale, swollen, or – in cases of reperfusion – eosinophilic neurons; these changes may be visible after 4 hours [88]. After about 12 hours, neutrophils infiltrate the tissue, and when approximately two days has passed, macrophages join; the macrophages can remain for months while scavenging tissue debris [88]. In tandem, reactive astrocytes proliferate at the infarct rim; the combined scavenging and gliosis results in a fluid filled space lined by astrocytes [88].

Signs of neovascularisation such as hyperplastic endothelium may become visible at the infarct rim after about five days [88]. Studies on human infarct tissue indicate increased levels of VEGF [134], increased vascular density [135], and immunohistochemical signs of newly formed capillaries [136]. While potentially positive for the prognosis, the destabilising effects of angiogenesis may also come with negative effects, such as increased vascular permeability and compromised blood brain barrier integrity [133].

During an acute infarct, the influence of collateral blood flow results in a successive gradient between normally perfused tissue and the severely ischaemic infarct core [132]. Despite this gradient, the typical end result appears to be rather dichotomous in the cortex from a histopathological perspective; old grey matter infarcts tend to have only a short distance between completely infarcted tissue and normally appearing tissue [137, 138][1]. The matter of the white matter is more of a grey zone: Complete white matter infarcts may be surrounded by large incomplete infarcts – areas where some, but not all parenchyma has perished, resulting in a rarefaction of the tissue rather than a complete loss [139].

In addition to focal ischaemia, global ischaemia caused by cardiac arrest with return of spontaneous circulation may result in selective neuronal death or complete infarcts [88]. Selectively vulnerable regions include hippocampus, neocortex, basal ganglia, thalamus, and cerebellum [88, 140]. The regional distribution of the injury is further determined by the vascular anatomy, leaving the border zones extra susceptible.

Border zone infarcts may also occur in a context of haemodynamic failure and arterial stenosis [23]. Such haemodynamic failure typically results in internal watershed infarcts or combined internal and external infarcts [22]. Isolated external border zone infarcts are not as strongly associated with haemodynamic failure; in these cases, embolism constitute a plausible aetiology [22, 141], although hypoperfusion could contribute by impairing washout of emboli [23].

[1] These findings were noteworthy as the authors performed their studies to examine the histopathological correlates of chronically reduced cerebral blood flow surrounding complete infarcts on imaging. Based on their results, they suggested that the reduction may represent hypometabolism due to disconnected and functionally impaired neurons, which would be invisible when viewed under a brightfield microscope.

Neuropathological corelates of chronic hypoperfusion

The main setting wherein chronic hypoperfusion is thought to contribute to neuropathological alterations is white matter pathology. White matter pathology can also occur in a context of neurodegenerative disease, wherein neuronal degeneration precedes degeneration of axons and myelin; these aetiologies can be difficult to separate, although some indications may be provided by the regional distribution of the lesions and by the state of the adjacent cortex [139]. In this thesis, I refer to the white matter pathology thought to be caused by cerebrovascular disease as 'white matter disease'.

White matter disease is neuropathologically evident as tissue rarefaction caused by loss of myelin, axons, and oligodendrocytes, and reactive gliosis; this pathology is often accompanied by widening of the perivascular space [142, 143]. White matter disease is associated with small vessel pathology, including arteriolosclerosis [143, 144], CAA [119, 120], and – possibly – arteriolar tortuosity [126]. With some heterogeneity, white matter disease represents a neuropathological correlate of white matter hyperintensities [145]. It affects deep or periventricular regions with relative sparing of the subcortical U-fibres [146], and is usually the most severe in the frontal lobe [147]. Deep white matter, such as the centrum semiovale, is thought to be particularly vulnerable to hypoperfusion due to its location within the internal border zone [22]. Biochemical findings suggestive of a hypoperfused state in white matter disease include increased levels of HIF1 [110] and a reduced MAG/PLP1 quotient [112, 148]; the latter finding further correlated with increased levels of VEGF [148]. Of note, the VEGF levels were positively associated with increased vascular density, indicating that the VEGF signalling could evoke angiogenesis [148]. Despite these findings, however, the vascular density in white matter disease is generally unaffected [110, 148, 149] or decreased [139, 150, 151].

Other microvascular features that have been associated with white matter disease include increased capillary diameters potentially suggestive of compensatory vasodilation [150], presence of string vessels [150, 152] that may indicate ongoing capillary regression [121], as well as other abnormalities regarding the capillary trajectory, morphology, and basement membrane [150, 152]. Neuropathological findings suggestive of affected blood brain barrier is implied by the presence of plasma proteins in the brain parenchyma [152, 153].

Another brain region where chronic hypoperfusion has been proposed as one potential aetiology is hippocampal sclerosis [154], which connotes hippocampal neuronal loss in CA1 and subiculum beyond what could be explained by any co-occurring Alzheimer's disease neuropathologic change [155]. However, hippocampal sclerosis has a heterogeneous and incompletely understood aetiology, including also neurodegenerative and age-related conditions [154-156].

The effects of chronic hypoperfusion on the neocortex, either direct or secondary to remote injury, are incompletely understood. The blood flow to the neocortex appears to be less vulnerable to haemodynamic alterations than the blood flow to other brain regions. While the deep grey matter may be more susceptible to hypertensive injury due to the direct transition from major arteries to penetrants, and the white matter may be more sensitive to hypoperfusion due to its long end arteries, the cortex could represent the 'Goldilocks zone' wherein the perfusion pressure is 'just right' [157][2]. In a context of mild reductions in cortical blood flow that can be observed in, for example, patients with dementia (blood flow reduced by up to 30% [158, 159] as compared to infarct and penumbra thresholds, which have been estimated to occur at 20–40% of normal blood flow [132, 160]), neurons maintain their ion gradients and ability to de- and repolarise [161]. However, the mechanisms and long-term consequences of milder reductions in cortical blood flow are incompletely understood, and there are no clear histopathological findings that are considered suggestive of chronic hypoperfusion in the neocortex. Still, this may not be sufficient to conclude that the neocortex is impervious to the effects of chronic hypoperfusion on the brain (see following sections).

Vascular dementia

The cumulative burden of vascular brain injury can result in cognitive impairment and dementia. Clinically, vascular dementia (VaD) can be classified as multi-infarct dementia associated with large vessel disease, subcortical ischaemic VaD associated with small vessel disease, post stroke dementia referring to dementia developing within six months of a stroke, and mixed dementia [162]. Overall, the pathology with the largest impact on cognitive impairment is considered to be small vessel disease [163], including (but not limited to) arteriolosclerosis and CAA [143, 164-166]. It has been proposed that vascular lesions tend to accumulate according to a specific chronology, beginning with alterations of the vessel wall (such as arteriolosclerosis), followed by widening of the perivascular space and white matter disease, while infarcts may occur as a late or independent contributor to cognitive impairment [143]. At some point in this progression, the vascular brain injury affects

[2] This phenomenon has been proposed to have an evolutionary basis. The primitive brain can be likened to a tube – in humans partially preserved in the brainstem, diencephalon, and basal ganglia. In contrast, the human cerebral hemispheres have expanded far beyond the rest of the brain, representing evolutionary new structures. The primitive brain is supplied by penetrants from the base of the brain that originate directly from the cerebral arteries, constituting a high-pressure system. In contrast, the arteries that supply the hemispheres have elongated correspondingly and given rise to an expansive arteriolar network wherein the prefusion pressure decreases, thus creating a low-pressure system. This model has been referred to as 'the ambibaric brain' and proposed to underly regional variations in vulnerability to hypertension and hypotension [157].

cognition; while there is no specific threshold for when it occurs, certain neuropathological findings can indicate the likelihood that cerebrovascular disease contributed to cognitive impairment [166].

As the term subcortical ischaemic VaD implies, small vessel disease mainly affects subcortical regions, including white matter and deep grey matter. The cortical effects of subcortical ischaemic VaD are less well understood. While the cortex is affected by histopathologically evident lesions in the VaD spectrum as a whole – large infarcts, microinfarcts, hippocampal sclerosis [167] – there are also indications that the cortex may be more diffusely affected in VaD. Imaging studies demonstrate decreases in cerebral blood flow involving both the cortex and the white matter, more prominently in anterior brain regions, with relative sparing of the occipital lobe [158, 168, 169]. The oxygen extraction fraction has been reported to be increased solely in the white matter [168] and the cortically decreased blood flow to correlate with white matter lesions [158], indicating that the cortical reduction in blood flow may have secondary to white matter disease.

In contrast, a neuropathological study reported that the MAG/PLP1 quotient measured in the frontal cortex was reduced in VaD, thus indicating chronic hypoperfusion [170] (the same method has been applied to examine hypoperfusion in the white matter [112, 148]). In the study that examined the cortical MAG/PLP1 quotient, VEGF exhibited an inconclusive tendency towards being increased compared to controls [170]. Regarding the cortical vascular density, studies that investigated this parameter found a tendency towards a decrease – the authors examined either cases with VaD [150] or cases with white matter disease regardless of cognitive status [171]. While not concerning the cortex directly, a noteworthy exception was the white matter in post stroke dementia, wherein the vascular density was increased, perhaps indicating that the vascular plasticity is affected by the chronicity of the disease [150]. Other immunohistochemical and biochemical studies on the cortex in VaD have described parenchymal deposits of plasma proteins around capillaries [172] and altered levels of synaptic proteins, potentially suggestive of synaptic loss and a compensatory restorative response [173]. Finally, proteomic analysis of the temporal cortex demonstrated upregulation of multiple pathways that could indicate hypoperfusion, blood brain barrier leakage, inflammation, and hypometabolism, paralleled by upregulation of protective pathways [174].

Neurodegenerative diseases

This section covers the neurodegenerative diseases studied in this thesis, with focus on some alterations in blood flow and vasculature that have been described in these conditions.

Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia, characterised neuropathologically by progressive accumulation of amyloid plaques and neurofibrillary tangles [175]. It has been proposed that the neuropathologic findings be referred to as Alzheimer's disease neuropathologic change (ADNC) to separate them from the clinical presentation [155].

Potential roles of vascular dysfunction in the development of AD include impaired clearance of amyloid beta due to dysfunctional interstitial fluid drainage [25], and experimental findings of increased amyloid beta generation under hypoperfusive conditions [176]. Cerebrovascular disease frequently co-occurs with ADNC [177] and it has been debated to what extent vascular risk factors contribute to its development [178]; however, AD can likely develop independently of such risk factors [179]. A recent meta-analysis and one neuropathological study further indicated that hypertension late in life (mild diastolic hypertension) was associated with a reduced risk of AD, although at the price of worsened small vessel disease [180, 181].

Nonetheless, AD is associated with vascular dysfunction that is likely intrinsic to the disease. Imaging studies have demonstrated reduced cerebral blood flow in AD that typically starts in the medial parietal cortex and later progresses to the other lobes [169]; an increased cortical oxygen extraction fraction has also been observed [168]. CAA occurs in 90% of patients with AD and may contribute to vascular brain injury in this setting as well as when it occurs independently. In addition to the structural alterations associated with CAA, functional alterations could also mediate hypoperfusion in AD. Amyloid beta can induce vasoconstriction of microvessels [182, 183], either directly or by evoking increased levels of the vasoconstrictor endothelin 1 [184], potentially due to upregulation of amyloid beta degrading enzymes that also produce endothelin 1 [185]. Biochemical analyses indicate cortical hypoperfusion (reduced MAG/PLP1 quotient), and the reduced MAG/PLP1 quotient was associated with increased levels of endothelin 1 [170].

Another aspect of amyloid beta concerns its interaction with VEGF. Parenchymal VEGF levels are increased in AD [186], more so than in VaD [170], potentially suggesting that amyloid beta can upregulate the production of VEGF beyond physiological requirements [170]. In tandem, however, VEGF can be sequestered in amyloid plaques [187], and amyloid beta may act as a VEGF antagonist [188], thus potentially reducing VEGF availability. One study that measured these parameters simultaneously indicated that the increase in VEGF was not accompanied by a corresponding increase in vascular density [170]. In fact, most studies reported a decreased vascular density in AD, although the results vary in this regard [115].

Biochemical signs indicative of compromised blood brain barrier integrity in AD include increased parenchymal fibrinogen [189] and reduced levels of tight junction

proteins [190] in the cortex. Other vascular abnormalities that have been associated with AD include increased thickening of the capillary basement membrane [191], abnormal capillary morphology [192], and increased capillary string vessels in the white matter [193].

Lewy body disease

Lewy body diseases (LBD) are synucleinopathies wherein alpha-synuclein aggregates into Lewy bodies and Lewy neurites [175, 194]. Clinical manifestations include dementia with Lewy bodies, Parkinson's disease with dementia, and Parkinson's disease [195]. Lewy body pathology frequently co-occur with ADNC, although the underlying mechanism of this co-occurrence is unknown [195].

Imaging studies of patients with clinically manifest LBD indicate reduced cerebral blood flow with predilection for the occipital lobes [196]; however, more widespread reductions in blood flow may also occur [159]. One study that compared the cerebral blood flow in patients with AD, dementia with Lewy bodies, and behavioural variant of frontotemporal dementia found it to be the most decreased in dementia with Lewy bodies throughout the brain [159]. A potential histopathological manifestation of reduced occipital blood flow in LBD is white matter disease (spongiform change and gliosis) with corresponding predilection for the occipital lobes [197].

One possible reason for reduced blood flow in LBD is intrinsic cholinergic denervation of the vasculature. Nucleus basalis of Meynert provides cholinergic innervation of neurons as well as intraparenchymal blood vessels [29, 30]. Cholinergic denervation with subsequent impaired vasodilation and hypoperfusion has been proposed as a contributory mechanism in the pathogenesis of AD – and other conditions associated with cholinergic deficit [198]. LBD is associated with degeneration of nucleus basalis of Meynert [199] and the cortex in LBD may exhibit marked cholinergic loss, as indicated by reduced acetylcholine esterase activity; the deficit was more pronounced than in AD [200]. Likewise, positron emission tomography of patients with clinically manifest LBD indicated cortical cholinergic deficit with a predilection for the occipital lobes [201], similar to the distribution of reduced blood flow demonstrated in other studies. While it does not prove the underlying mechanism, treatment with acetylcholine esterase inhibitors improves cerebral blood flow [202, 203].

Another potential mechanism by which LBD could be associated with recurrent hypoperfusion is cardiovascular autonomic dysfunction. Such dysfunction, including orthostatic hypotension, is a frequent feature of LBD compared to other neurodegenerative diseases and VaD [204, 205] (although it can occur in AD, frontotemporal dementia, and VaD as well [204-206]). In LBD, a potential underlying mechanism constitutes sympathetic denervation due to alpha-synuclein

aggregates within the peripheral nervous system [43, 207]. As with other forms of orthostatic hypotension, it is not known to what extent this condition can cause structural brain injury [208]. One study found that cases with LBD and symptoms indicative of orthostatic hypotension had increased small vessel disease burden and associated vascular brain injury when compared to LBD without such symptoms, indicating either that both pathologies contributed to and exacerbated orthostatic hypotension, or that the orthostatic hypotension contributed to the development of the vascular pathology [209].

A third component to consider in the context of LBD is the potential interaction between alpha-synuclein and VEGF. One biochemical study on the cortex of cases with LBD reported signs of chronic hypoperfusion (reduced MAG/PLP1 quotient), reduced vascular density, reduced VEGF levels, and an inverse relationship between VEGF and alpha-synuclein [210]. Based on these findings and experimental data, it was concluded that alpha-synuclein may inhibit normal VEGF expression, impair angiogenesis, and ultimately contribute to reduced capillary density with subsequent hypoperfusion [210]. The authors only found this combination of results in the occipital cortex [210]. Other studies, however, found cortical capillary alterations with a wider regional distribution, including reduced capillary density [150, 211], presence of capillary strings (indicating capillary regression) [150, 212], and increased capillary diameters (indicating compensatory vasodilation) [150, 211]. In contrast to these findings, other studies focusing on substantia nigra reported increased levels of VEGF [213], increased vascular density [214], and immunohistochemical labelling indicative of newly formed blood vessels [215]; the latter study further reported similar findings in putamen. As such, the results regarding VEGF and angiogenesis in the LBD spectrum are not unanimous, although perhaps slightly more so if limiting the scope to the forebrain and cortex.

From a neuropathological perspective, LBD has been independently associated with microbleeds, interpreted as a potential indicator of blood brain barrier dysfunction [216].

Frontotemporal lobar degeneration

Frontotemporal lobar degeneration (FTLD) is the neuropathological equivalent of frontotemporal dementia [175]. Frontotemporal dementia constitutes a heterogeneous spectrum of diseases, including behavioural variant frontotemporal dementia, primary progressive aphasia, corticobasal degeneration, progressive supranuclear palsy, and motor neuron disease, including amyotrophic lateral sclerosis [217]. FTLD can be categorised based on the proteins of the neuronal and glial inclusions that accompany the pathology. In a majority of the cases, the underlying protein is tau or transactive response DNA-binding protein (TDP) 43, and in a minority of the cases fused in sarcoma (FUS) [218, 219]. In rare cases, no specific protein is found other than those of the ubiquitin-proteasome system (UPS)

[218, 219]. The most common neuropathological diagnoses within the FTLD-tau spectrum are progressive supranuclear palsy, corticobasal degeneration, and Pick's disease; an example of a rare diagnosis (mentioned here as it will be brought up later) is glial globular tauopathy. FTLD-TDP, in turn, can be classified into type A, B, C, or D [220]; type E connotes a fifth type [221], although consensus has not been reached whether it is a separate type or part of type B [217]. The most frequent mutations causing FTLD are located within *MAPT*, (encodes tau, manifests as various diagnoses within the FTLD-tau group), *GRN* (encodes progranulin, causes FTLD-TDP type A), and *C9orf72* (chromosome 9 open reading frame 72, typically associated with FTLD-TDP type B) [222].

As in other neurodegenerative diseases, frontotemporal dementia is associated with reductions in cerebral blood flow [159, 169], which has been demonstrated to occur at a presymptomatic stage in genetic forms, mainly in the subgroup with *C9orf72* mutation [223]. While not associated with conventional vascular risk factors, recent studies on the pathophysiology of FTLD have demonstrated other vascular abnormalities in different diagnoses of this disease group. These include increased capillary density in familial glial globular tauopathy [224] and upregulated angiogenic pathways in FTLD-TDP [225, 226]. Furthermore, another study on the cortical gene expression in FTLD-TDP type A with *GRN* mutation indicated that neurovascular dysfunction may be an important feature in the pathogenesis of this disease [227]. Findings with vascular implications included HIF expression (*HIF3A*) indicative of hypoxia, reduced VEGF signalling (normally functioning progranulin induces VEGF), increased expression of VEGF-independent angiogenic factors (perhaps compensatory), increased vascular density, and compromised blood brain barrier integrity [227]. It is not known to what extent these findings apply to other diagnoses of the FTLD group.

Ageing

Ageing is important to account for in neuropathological studies. From a cellular point of view, ageing is associated with cumulative genomic damage that induces cellular senescence and other responses to injury; ultimately, these responses may become deleterious by contributing to stem cell exhaustion, impaired cellular interactions, and chronic inflammation [228, 229]. As such, ageing becomes a risk factor for multiple pathologies – including vascular and neurodegenerative – by exerting an independent influence on the function and structure of tissues and organs [228, 229]. The ageing process can be further accelerated by acquired pathologies such as obesity, diabetes, and hypertension [230], but may also be decelerated by other parameters, such as physical exercise [231].

The importance of different risk factors further varies across the age span. For example, hypertension in mid-life is considered an important risk factor for future cerebrovascular disease [232]. In contrast, arteriolosclerosis, typically associated with hypertension, may no longer be associated with this condition in the elderly population [104]. Perhaps individuals who live longer have fewer risk factors, ultimately making ageing itself the primary influence of vascular function and structure.

Potential vascular manifestations of ageing include stiffening of elastic arteries [95], arteriolar tortuosity [126], arteriolosclerosis [104], increased thickening of the basement membrane [233], arteriolar and possibly capillary rarefaction [95], affected angiogenic capacity [95], reduced cerebral blood flow [169, 234], and affected integrity of the blood brain barrier [235, 236]. These manifestations can overlap with alterations associated with vascular and neurodegenerative diseases – there may only be so many different patterns a tissue can demonstrate in response to injury.

Previous studies on raspberry-like microvascular entities

In this 'jungle' of small vessel alterations that can affect the brain grows a specific microvascular entity that we refer to as 'raspberries'.

As we began to examine these structures more closely, we also learned of observations made by other authors on similar vascular entities. Some of the earliest observations may be the ones by Alzheimer and Cerletti in the beginning of the 20th century [237, 238][3]. Cerletti systematically examined structures referred to as 'Gefäßknäuelbildungen', 'Gefäßbündel', and 'Gefäßgeflechte' [238]; these terms were later translated as 'glomerular loop formations', 'vascular bundles', and 'vascular wickerworks' by Swedish neuropathologist Ove Hassler in the 1960s [239]. Hassler took an interest in these vascular entities and examined them by microangiography in a non-selected cohort of autopsied individuals [240], expanding the concept beyond the cases of malaria wherein Cerletti had performed the majority of his observations.

Among these three vascular abnormalities, glomerular loop formations likely correspond to the arteriolar tortuosity observed by other authors in mainly white and deep grey matter [121, 122]. The wickerworks and bundles, in turn, were restricted to grey matter, including the cortex [239]. 'Vascular wickerworks' connoted at least two arterioles that ran in parallel while intertwining like a rope, whereas 'vascular

[3] This was prior to and – likely – unrelated to their later roles in the characterisation of Alzheimer's disease and the development of electroconvulsive therapy, respectively.

bundles' referred to at least four arterioles running in parallel [239]. Such formations cut transversally would likely correspond to a raspberry.

In his studies, Hassler noted that the formations increased with advancing age, and further saw a tendency towards an association with cerebral atherosclerosis and cardiac hypertrophy in his descriptive data [240]. Initial indications of an association with dementia were not supported in a subsequent study [241].

From the 1960s two the 1980s, other authors described similar formations of cortical blood vessels running in parallel, identified when examining the vasculature by microangiography or by the study of vascular casts. In several cases, these were secondary findings in studies on the cortical vascular anatomy [21, 32, 33, 242]. Some of these authors specified the location of these formations to the branching segments of penetrating arterioles as follows: A penetrating arteriole would sometimes give off multiple branches while still remaining within a single perivascular space, and the arterioles would run in parallel within this space for some distance, before the branches took off in individual directions [21, 32, 33]. This pattern was found mainly in the intermediate and deep levels of penetrant branching but was also observed within the branches originating from the penetrants [21]. As an additional feature, the blood vessels constituting the formations were frequently intertwined [32], thus resulting in formations with characteristics of both vascular bundles and wickerworks (Fig. 6).

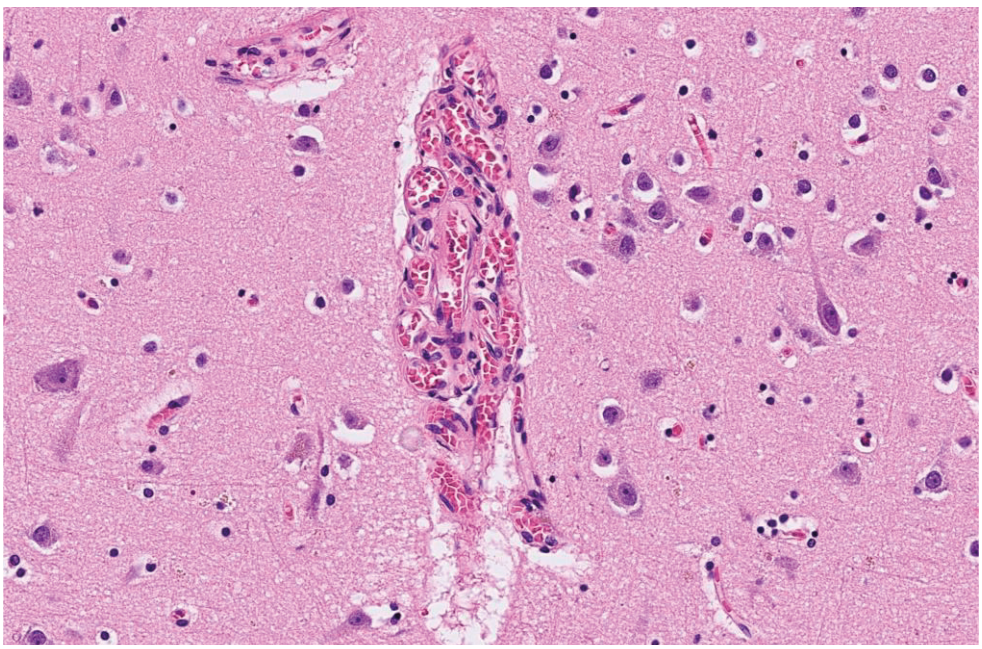

Fig. 6
Cortical vascular bundle with intertwining vessels.

Based on these findings, Cervos-Navarro et al. characterised the ultrastructure of the formations by the application of electron microscopy [243]. The authors concluded that the formations, here referred to as 'vascular convolutes', were exclusively arteriolar and confined within a common perivascular space. Like in some of the aforementioned studies on the cortical vascular anatomy, Cervos-Navarro et al. described an increasing prevalence with advancing age.

In the early 2000s, Arsene et al. aimed to examine 'vascular convolutes' with brightfield microscopy [244]. Some of the vascular formations which they observed were indeed similar to and overlapping with raspberries, although they did not provide an exact definition adapted for the two-dimensional context. In their second study on this topic, the authors reported an inverse relationship with haemorrhagic transformation of infarcts [245].

In 2023, Ighodaro et al. examined formations termed 'multilumen vascular profiles' that were defined in the same manner as raspberries [246]. By quantifying these structures in the frontal cortex, the authors identified a statistically significant increase with advancing age and a potential association with self-reported brain trauma. Immunohistochemical labelling of astrocytes and microglia did not indicate increased glial reactivity around the vessels.

Regarding theories on why these microvascular entities would form, neovascularisation [239, 244], arteriolar elongation in response to hypoxia [243, 244], or an adaptation to hypertension that reduces the risk of haemorrhagic transformation of infarcts [245] have been proposed.

In summary, cortical structures with some resemblance to raspberries – and likely overlap – have been observed by other authors throughout the years. Even though the findings have been *en passant* in some cases, other attempts have been made to characterise them systematically, as briefly reviewed above. However, much of the clinicopathological context of raspberries remains unknown, with the only recurring finding being an increasing density with advancing age, which, until the study by Ighodaro et al. [246], was mainly a descriptive finding unaccompanied by statistical analyses.

Our own initial observations indicated that the number of raspberries varied considerably between individuals of similar age. We wanted to explore this variation further. As may be apparent from the background leading up to this section, our hypothesis was that raspberries represent a form of neovascularisation occurring as a result of chronic or recurrent hypoperfusion, wherein the blood flow remains above the infarct threshold. While raspberries occur in other parts of the brain as well, the fact that they are well as frequent in the cortex as in other regions interested me. The consequences of hypoperfusion on the cortex in the context of cerebrovascular or neurodegenerative diseases are incompletely understood. If raspberries were to represent a morphological sign of cortical hypoperfusion, this knowledge might be of value in neuropathological diagnostics, and could teach us

something about how different parts of the brain react to hypoperfusion. This thesis attempts to put raspberries within a clinicopathological context beyond age, in the hope of guiding further research on their pathogenesis and potential consequences.

Aim

The aim of this thesis was to examine and specify the clinicopathological context of raspberries. Our hypothesis was that raspberries form as a reaction to chronic or recurrent hypoperfusion; this has guided the study designs. Later parts of the project further aimed at examining to what extent raspberries are associated with age, and to follow up on initial findings from earlier exploratory studies and pilot studies.

In Paper I, we examined the raspberry density in vascular dementia, frontotemporal lobar degeneration, Alzheimer's disease, and control cases. These cases represented advanced cerebrovascular pathology and neurodegenerative disease.

In Paper II, we quantified raspberries in relation to individual findings of vascular disease and clinical conditions that might be indicative of hypoperfusion, in an attempt to see whether differences in raspberry density could be associated with less advanced neuropathological findings.

Paper III tested the findings from Paper II in an independent study sample.

Paper IV examined raspberries in relation to age while accounting for potential confounding factors, in an attempt to establish to what extent this relationship can be considered independent.

In Paper V, we re-examined the raspberry density in frontotemporal lobar degeneration based on earlier indications of an increased raspberry density within this spectrum. We also added to the previous data on raspberry density in neurodegenerative diseases by examining raspberries in relation to Lewy body disease.

Methods

Study population

This was a retrospective, cross-sectional project based on adult individuals who had undergone a postmortem diagnostic neuropathological examination. The neuropathological examinations were performed at the Department of Clinical Pathology (Sektion Klinisk Patologi) in Lund, which is part of Clinical Genetics, Pathology, and Molecular Diagnostics, Region Skane, Sweden. The patients were referred to a neuropathological examination to diagnose dementia, to diagnose other diseases affecting the brain, or as part of a broader examination to establish cause of death in cases without a prior history of neurological or psychiatric illness. A majority of the patients underwent a full clinical autopsy prior to the neuropathological diagnostics, including macroscopic examination and weighing of the internal organs, as well as tissue sampling for histopathological diagnostics, the extent of which depended on the clinical context. In a minority of the cases, the autopsy was restricted to a neuropathological examination only. The total time period from which cases were included encompassed January 1993–April 2023; most patients were included from 2010 and onwards (Fig. 7).

The data described and analysed in the project was collected by examining archival formalin-fixed, paraffin-embedded brain tissue – for quantification of raspberries – and autopsy reports and medical records – for retrieval of autopsy findings and clinical data.

Neuropathological procedure

Since the project was retrospective and based on biological material collected and records established during clinical practice, the diagnostic neuropathological examinations had already been performed and reported. The general features of such a procedure are outlined here.

The brain (forebrain, brainstem, and cerebellum) is fixed for several weeks. A macroscopic examination follows, wherein signs of atherosclerosis in the arteries at the base of the brain are noted, as are signs of brain atrophy or focal lesions. The forebrain is cut in the coronal plane into sections 1–1.5-cm thick; the brainstem and

cerebellum are cut horizontally into sections 0.5–1-cm thick. The subsequent sampling typically includes tissue blocks from the cerebral lobes (neocortex and subjacent white matter), hippocampus, basal ganglia, cerebellum, brainstem (at the level of substantia nigra, locus coeruleus, and medulla oblongata), and one or several bi-hemispherical sections from the forebrain. After dehydration and paraffin-embedding, the tissue sections are cut 6-µm thick if staining with haematoxylin and eosin or special stains is planned, and 3-µm thick for immunohistochemistry. All tissue blocks are stained routinely with haematoxylin and eosin. Luxol fast blue is applied to examine white matter pathology. Alkaline Congo red or immunohistochemical labelling of amyloid beta is applied to assess CAA. Tangles and neuritic plaques are assessed with Gallyas silver stain or immunohistochemical labelling of tau. Aggregates involving other proteins (such as alpha-synuclein and TDP-43) are assessed by immunohistochemistry.

The neuropathological diagnosis of VaD is based on multiple ischaemic lesions and vessel wall pathology in absence of significant neurodegenerative disease [247, 248]. ADNC, LBD, and FTLD are classified according to consensus criteria [249-252], and updates [155, 218, 219, 253]. While the updates often build upon the previous consensus criteria, this may add some heterogeneity to the material when the inclusion period is long. However, most of the patients included in this thesis underwent neuropathological diagnostics after 2010 (Fig. 7). Another limitation is associated with including patients who underwent a neuropathological examination for reasons other than diagnosing dementia, since clinically motivated deviations from the standard procedure outlined above will occur in some of these cases. Such deviations could reduce the chances of detecting subclinical pathology, which in turn could exert a hidden influence on the raspberry density. However, including individuals from a broader context than patients affected by dementia may provide a better notion of how raspberries are distributed also in patient groups with less advanced neuropathology. Since we have no previous baseline raspberry density in the underlying population to compare to, such cases are also important to include as they may serve as controls.

Data from medical records and autopsy reports

Clinical data and autopsy findings were retrieved by assessment of medical records and autopsy reports. The autopsy reports were accessed via the two systems Sympathy (prior to April 2019) and LIMS RS (from April 2019 and onwards); these are digital systems wherein all autopsies performed at the Department of Clinical Pathology are documented. The medical records were accessed via the digital system Melior, wherein records of public specialist healthcare in Region Skane are kept.

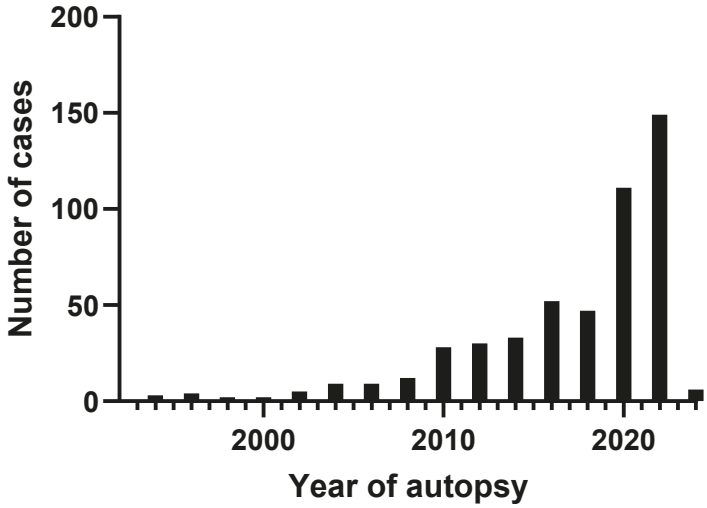

Fig. 7
Histogram depicting the total time period from which cases were included in the project (January 1993–April 2023; n = 511).

In Paper I, subjects were included based on neuropathological diagnosis in combination with clinically diagnosed dementia. In Sweden, diagnoses are coded according to ICD-10 [254], in which cognitive impairment can be diagnosed as 'mild cognitive impairment' or 'dementia' depending on its severity. When assessed within the course of this project, data on cognitive status was based on clinically diagnosed mild cognitive impairment and dementia, regardless of which criteria that were applied to establish the diagnoses.

In Paper II, data retrieved from autopsy reports included aortic atherosclerosis, cerebral atherosclerosis (as assessed in the basal cerebral arteries), cerebral small vessel disease (arteriolosclerosis, lipohyalinosis, and small vessel atherosclerosis [255][4]), CAA, cerebral infarcts, and white matter disease; data from clinical records included hypertension, diabetes mellitus, orthostatic hypotension, heart failure, and acute circulatory failure. In Paper III, the inclusion of data from medical records and autopsy reports were limited to cerebral atherosclerosis and acute circulatory failure; this approach was based on the results of Paper II.

Paper IV included individuals from Paper II and III as well as newly included individuals. The autopsy reports and clinical records were assessed – or re-assessed

[4] Lipohyalinosis connotes a small vessel disease that partially overlaps with arteriolosclerosis; in later papers, the definition of 'small vessel disease' was altered to connote arteriolosclerosis alone.

– to further characterise the study sample regarding neuropathological diagnosis, and to add data on cardiac hypertrophy and nephrosclerosis to enable ordinal ranking of hypertension severity. In contrast to Paper I, neurodegenerative disease was categorised based on the neuropathological examination alone and did not require clinically verified cognitive impairment. This altered threshold was deemed preferable, since the aim was to exclude that neurodegenerative pathology affected the relationship between raspberries and age.

In Paper V, cases were included based on a neuropathological diagnosis of FTLD or LBD. Clinical data on these patients has not yet been retrieved.

A general limitation of retrospective studies is that the clinical data acquired from the medical records varies in quantity and quality; all patients are not examined in the same way and at the same time in relation to death, and the follow up over time varies. In Paper II, an attempt was made to address this limitation by validating the diagnoses of acute circulatory failure (also applied in Paper III) [256, 257], orthostatic hypotension [258], and heart failure [259] according to consensus criteria, although this approach is also limited by the retrospective design. Acquiring data in retrospect from autopsy reports is associated with similar limitations. For example, individual findings of cerebrovascular disease and ischaemic lesions are currently not graded according to a specific schedule, thus limiting the possibility to extract semi-quantifiable data. Such limitations may be partially overcome by expanding the study sample and may be further handled, or visualised, by using a wide range of available data to characterise the sample.

Quantification of raspberries

A raspberry is a histopathological finding defined as a minimum of three transversally sectioned vascular lumen surrounded by a common perivascular space. The definition has remained throughout the course of the project, although the method of quantification has undergone some variation. Typically, the quantification is performed on haematoxylin and eosin-stained tissue sections of the neocortex mounted on standard-sized glass slides. Throughout the text, I will use the term ‘raspberry density’ regardless of the unit of measurement, and specify when relevant.

In the first part of the project, the raspberries were counted by examining the tissue sections under a bright-field microscope. A 20-mm distance was measured at the cortical surface, and the raspberries in the underlying cortex were counted. The unit of measurement was raspberries/cm. The raspberry density of an individual brain was defined as the average raspberry density of the frontal, temporal, and parietal and/or occipital cortex.

In Paper II–V, the tissue sections were examined digitally in either Aperio ImageScope (cases included prior to April 2019) or Sectra IDS7 (cases included from April 2019 and onwards). In each tissue section, the cortical area was measured, and the raspberries within the delineated area were marked (Fig. 8). The new unit of measurement became raspberries/cm². With some exceptions, the raspberry density of an individual brain was estimated by examining a tissue section from the anterior frontal cortex (corresponding to Brodmann area 10, 9, or 46).

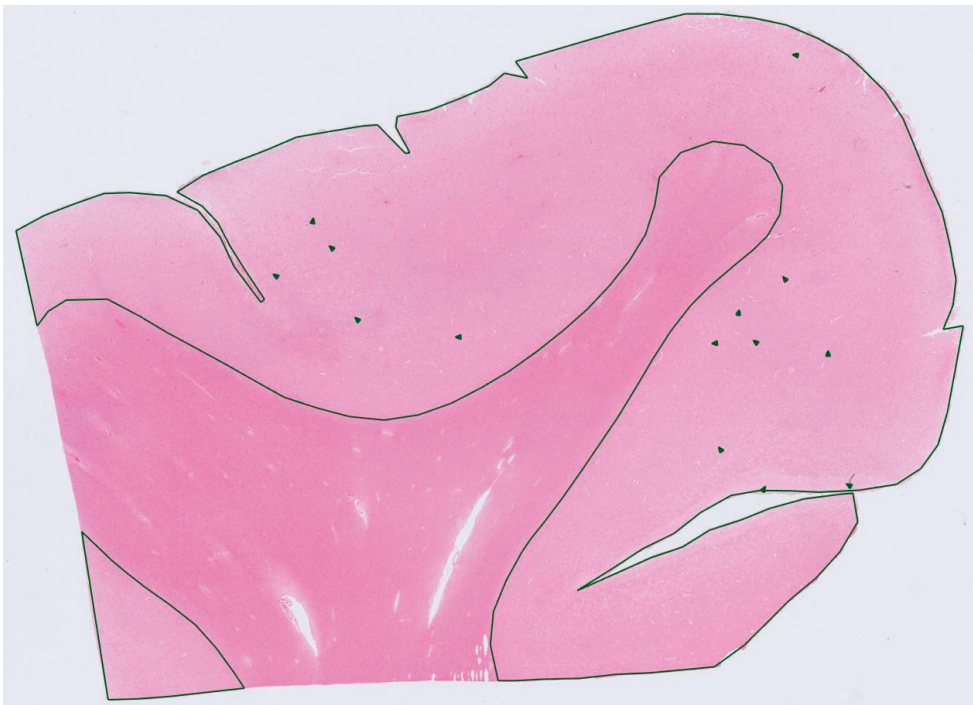

Fig. 8
Overview of a tissue section from the frontal cortex. The cortical area has been delineated and the raspberries have been marked.

In Paper II and III, this new unit of measurement was standardised for variations in brain weight. Weight standardisation was performed under the assumption that altered brain volume caused by atrophy or oedema – and manifested as an abnormal brain weight – could affect the raspberry density in a way that did not reflect a change in the absolute number of raspberries. The standardisation was achieved by multiplying the raspberry density by the quotient of the individual brain weight divided by the sex specific mean brain weight [260]:

> [Raspberries/cm²] × ([Individual brain weight] ÷ [Sex-specific mean brain weight])

In Paper IV and V, unstandardised raspberry density was used. Standardising for brain weight prior to performing the statistical analyses may be a valid approach in a setting wherein atrophy can be assumed to be random in relation to the dependent variable. In Paper IV and V, this was deemed not to be the case. If brain weight were to correlate with the dependent variable, weight standardisation prior to the statistical analysis could potentially hide an atrophy-independent component in the variation in raspberry density; other approaches may be preferable in such a scenario. In Paper IV, brain weight was included as a variable in the multiple linear regression models. In Paper V, the relationship between raspberry density and brain weight was assessed in a subgroup analysis once the primary statistical analysis had been performed. Brain atrophy is likely relevant to account for in some way, but the best approach may differ depending on the context. Methods other than standardisation for brain weight may need to be considered in some situations (see Discussion).

The inter-rater variability of raspberry quantification was examined in Paper I on haematoxylin and eosin-stained tissue sections from the frontal lobe (cortex). Since raspberry density is a continuous variable, the results of two independent raters were compared in a Bland–Altman plot. This approach does not come with a specific cutoff, but the results were deemed sufficiently reliable to allow further comparisons without changing the definition of raspberries.

In Paper I, an alternative method of raspberry quantification was applied: A subset of the tissue blocks underwent sectioning and immunohistochemical labelling for collagen 4 (Fig. 9). The raspberry density in these tissue sections was compared to that of the corresponding haematoxylin and eosin-stained sections. The immunohistochemical procedure was performed by biomedical analysts; the collagen 4 antibodies were already in clinical use at the laboratory. Collagen 4 is abundant in the basement membrane and labelling it will clearly distinguish the blood vessels from the surrounding parenchyma. While the immunohistochemically labelled tissue sections revealed more raspberries than did haematoxylin and eosin – becoming a limitation if one wishes to establish the absolute raspberry density – it was still possible to identify relative differences based on haematoxylin and eosin. As such, assessment of tissue sections already stained for haematoxylin and eosin during the neuropathological diagnostic procedure remained the main method of raspberry quantification.

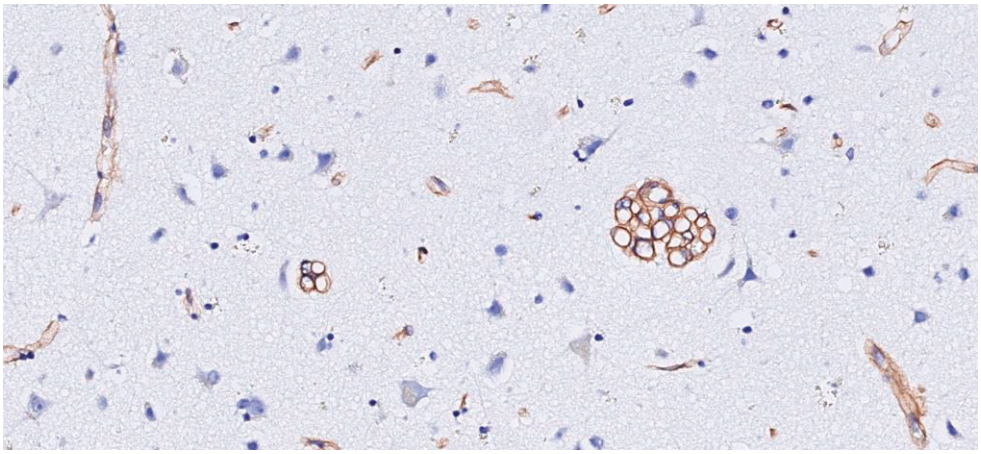

Fig. 9
Cortical raspberries immunohistochemically labelled for collagen 4.

Statistics

Statistical analyses were performed in IBM SPSS Statistics (different versions). Data were analysed using non-parametric or parametric tests depending on the group sizes and the distribution of the outcome variable. In some contexts, a combination of parametric and non-parametric testing was performed. Small groups or non-normally distributed data were preferably analysed with non-parametric tests (the Kruskal–Wallis test, Dunn's post hoc test, the Mann–Whitney test, the Friedman test, or the Wilcoxon signed ranks test). Large and normally distributed data sets were preferably analysed using parametric tests (independent samples t-test, simple linear regression, multiple linear regression, or ANOVA followed by post hoc tests). A P value of < 0.05 was considered statistically significant; Bonferroni correction for multiple tests was used when applicable. In two papers (Paper III and V), sample size calculations were performed in an attempt to estimate group sizes that would enable testing of the hypotheses with sufficient statistical power; such calculations were performed in Epitools [261]. Strategies to address confounding factors included either matching for age, sex, and examined brain region, or multiple linear regression.

Results

Overview

In total, the project was based on 511 individuals, some of whom were included in more than one study. When measured in the frontal cortex as raspberries/cm² without brain weight standardisation (possible in 500 cases), the mean raspberry density of the entire study sample was 6.6 raspberries/cm², the median raspberry density 5.0 raspberries/cm², and the total range 0–32.5 raspberries/cm² (median age 73; age range 20–97; 37% women; Fig. 10).

Some general features of raspberries

Some general features of raspberries have been reported in the papers; they are briefly summarised here while accompanied by some additional characteristics.

In many cases, raspberries only fulfil the minimum requirement of 3 lumen, in contrast to extreme cases, in which up to 20 lumen can be observed. The proportion of raspberries with many lumen may be higher in individuals with high raspberry density, such as in VaD (indicated in Paper I). The vascular lumen are generally no wider than 20 μm in diameter with many lumen being closer to 10 μm. The vessel walls tend to be labelled strongly and evenly by alpha-smooth muscle actin antibodies, indicating that most raspberries likely are arterioles (Fig. 11). Mild hyaline thickening of the vessel wall can occasionally be encountered. The distribution of raspberries within a tissue section is sometimes clusteriform rather than diffuse (Fig. 12); this may be partially dependent on the angle by which the cortex was cut, although I do not believe this to be a sufficient explanation for the full range of this pattern.

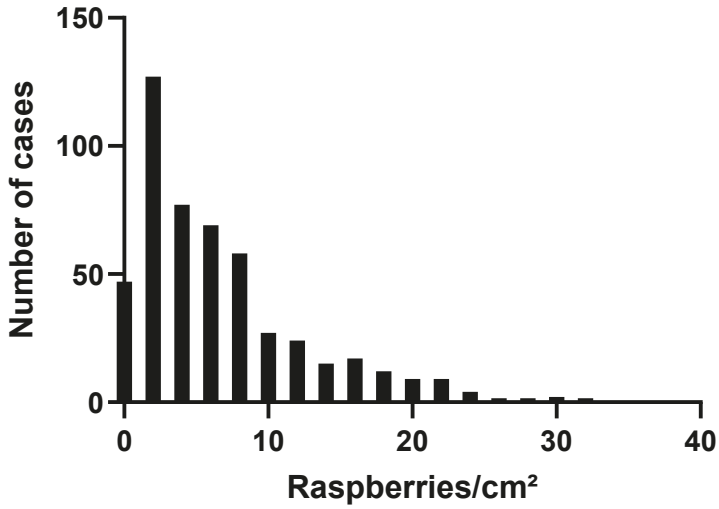

Fig. 10
Histogram depicting the variation in raspberry density (raspberries/cm²) as quantified in the frontal cortex without brain weight standardisation and regardless of underlying diagnosis (n = 500; median age 73; age range 20–97; 37% women).

Raspberries in relation to brain region

The regional distribution of raspberries was examined in Paper I and Paper III. In Paper I, the raspberry density was compared between the frontal, temporal, parietal, and occipital cortex across the entire study sample. The statistically significant finding was a higher raspberry density in the frontal cortex compared to the occipital cortex. In Paper III, the raspberry quantification was expanded to include the basal ganglia, hippocampus, pons, cerebellum, and hemispheric white matter (centrum semiovale). Raspberries were frequent in cerebral grey matter, contrasting with sparse findings in cerebellum and centrum semiovale. I have not found a clear predilection for the external border zones (unpublished pilot study), perhaps indicating that if raspberries are indeed caused by hypoperfusion, other mechanisms than hypotension in combination with impaired/overrun cerebral autoregulation may be at play. However, a systematic examination of the raspberry 'gradient' along the cerebral convexities in bi-hemispherical tissue sections from cases selected based on a clinical context of long-standing hypotension has not been performed.

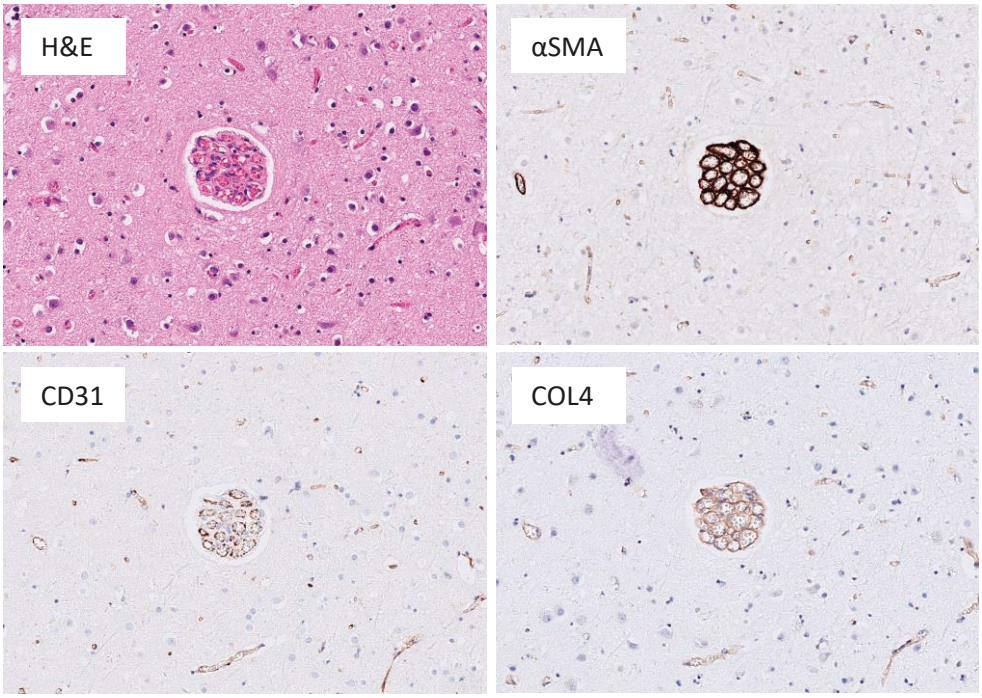

Fig. 11
Four shades of a cortical raspberry, including routine haematoxylin and eosin (H&E) staining, and immunohistochemical labelling for alpha-smooth muscle actin (αSMA), endothelial marker CD31, and basement membrane marker collagen 4 (COL4).

Demographics: Age and sex

The potential association between raspberry-like vascular entities and increasing age has been observed by several authors, although only one previous study verified the results in a statistical analysis [246]. We initially included age in the statistical analysis in Paper II and III (range: 46–97 years); these studies demonstrated a weak positive association that was statistically inconclusive. Given the findings of other authors, we designed Paper IV with the primary aim of examining the relationship between raspberries and age, including establishing to what extent this relationship could be considered independent. The results demonstrated an increasing raspberry density when tested with non-parametric methods as well as multiple linear regression models. The major increase in raspberry density appeared to occur in the 60–69 or 70–79-year interval; the raspberry density may have begun to increase to a lesser extent from a younger age, but this was not verified in the statistical analyses. The raspberry density variance was higher among elderly individuals,

although a few outliers with high raspberry densities occurred even in the 40–49-year interval.

The raspberry density according to sex was documented in Paper II and was somewhat higher in women (not statistically significant). In Paper IV, this pattern reappeared and now achieved statistical significance.

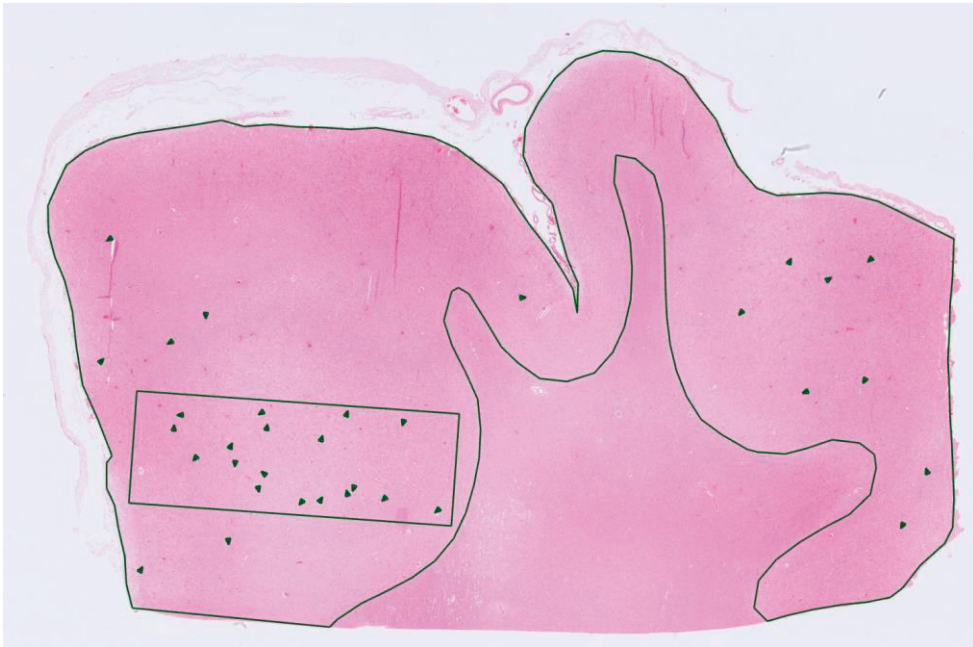

Fig. 12
Clusteriform distribution of raspberries in the frontal cortex.

Clinical data and autopsy findings

These comparisons were made mainly in Paper II, III, and IV (most individuals in Paper IV were also included in Paper II or III).

In Paper II, the raspberry density did not differ between individuals based on presence of clinical hypertension, diabetes mellitus, orthostatic hypotension, atrial fibrillation, chronic heart failure, or a history of acute circulatory failure, nor did it differ based on autopsy findings of aortic atherosclerosis, cerebral small vessel disease, CAA, cerebral infarcts, or white matter disease. There was, however, a statistically significant association with cerebral atherosclerosis; subjects with cerebral atherosclerosis had on average a higher raspberry density than those who were free of this pathology. Descriptive data indicated that within the group with

cerebral atherosclerosis, patients with a history of acute circulatory failure had an even higher raspberry density.

Due to the explorative nature of Paper II, the association between raspberry density and cerebral atherosclerosis was examined once more in an independent study sample (Paper III). Paper III also included acute circulatory failure, in line with the descriptive data from Paper II. The results of Paper III provided no indication that the raspberry density was affected by a history of acute circulatory failure; however, the results supported an association between raspberries and cerebral atherosclerosis, albeit weaker than previously indicated. The positive association between raspberry density and cerebral atherosclerosis was also observed in the multiple linear regression models in Paper IV (wherein the individuals from Paper II–III were included).

The multiple linear regression models applied in Paper IV further indicated a positive association between raspberry density and hypertension, but only when the clinical hypertension was subgrouped according to autopsy findings suggestive of hypertensive organ damage (cardiac hypertrophy in combination with either cerebral arteriolosclerosis or nephrosclerosis). As might be expected, non-hypertensive individuals (median age 65) were younger than hypertensive individuals; however, hypertensive individuals without organ damage were of a similar age as hypertensive individuals with organ damage (median age 74 and 74.5, respectively).

Paper IV also indicated an independent association between raspberry density and cardiac hypertrophy.

Vascular dementia and neurodegenerative diseases

The raspberry density in relation to VaD and/or neurodegenerative disease was examined as a primary aim in Paper I and Paper V. In Paper I, we compared the raspberry density between individuals with VaD, AD, FTLD, and control cases. In Paper V, we re-examined the raspberry density in FTLD in a larger study sample, while also examining LBD and control cases. In Paper I, a clinical diagnosis of dementia was required for inclusion in the disease groups; in Paper V, this was not a prerequisite for inclusion. TABLE

In Paper I, the raspberry density was the highest in VaD, which differed from all the other groups at a statistically significant level. The second highest raspberry density was observed in FTLD, followed by AD, and then control cases; these differences did not reach statistical significance. Later, a pilot study indicated an increased raspberry density in FTLD compared to control cases, whereas the raspberry density in AD now was similar to that in the control group. The increased raspberry density

in FTLD led us to examining the raspberry density in this disease group once more in a larger study sample (Paper V). The results demonstrated a higher raspberry density in FTLD compared to both LBD and controls at a statistically significant level; the LBD raspberry density was not indicated to differ from that in controls.

When examining the entire FTLD group, there was a statistically inconclusive tendency for a negative correlation between raspberry density and brain weight. Additional subgroup analyses of the FTLD group did not indicate differences in raspberry density based on underlying proteinopathy (tau, TDP-43, FUS, UPS, other/combined); neither was there any statistically significant differences between the neuropathological subtypes within the FTLD-tau or FTLD-TDP spectra. In the LBD group, descriptive data provided no indication of an increased raspberry density in patients with more severe (neocortical) LBD pathology or in cases with co-occurring ADNC; rather, these cases had a slightly lower raspberry density on average.

In Paper IV, the inclusion of data on neurodegenerative disease and VaD or vascular mild cognitive impairment aimed at excluding that such diagnoses affected the relation between raspberries and age. In this paper, the multiple linear regression models indicated a negative association between raspberry density and ADNC, thus contrasting the higher raspberry density observed in AD in Paper I.

Summary

The following variables demonstrated a statistically significant association with raspberry density which either remained after correction for multiple tests or was verified in an independent study sample: age, VaD, FTLD, and cerebral atherosclerosis. The following variables have been associated with raspberry density in an exploratory setting (not corrected for multiple tests): female sex, cardiac hypertrophy, hypertension subgrouped for organ damage, and ADNC.

Discussion

Raspberries and overlapping microvascular entities have attracted some attention from different authors throughout the years, from Alzheimer and Cerletti in the early 1900s, to Hassler in the 1960s, Cervos-Navarro et al. in the 1980s, and more contemporary observations by Arsene et al., Ighodaro et al., and us. Some previous attempts have been made to place these entities in a clinicopathological context, the recurring observation being an association with advancing age. However, many vascular and other pathological findings can be associated both with age as well as with acquired pathologies. As such, this study has attempted to narrow down the clinicopathological context of raspberries. Specifying this context is of value when directing the focus of future research on the pathogenesis, consequences, and clinical relevance of raspberries.

In the following sections, I have attempted to discuss the combined findings from the papers included in this thesis, while also addressing some topics that have not been examined directly in the papers.

Are raspberries vascular bundles? Are raspberries remodelled blood vessels or formed de novo?

This section focuses on the relationship between raspberries and a potential three-dimensional correlate – vascular bundles – that has been observed by other authors. Some potential mechanisms by which vascular bundles – and possibly raspberries – could form are also discussed. We have not yet addressed these questions directly, but I believe they may be relevant to reflect upon in this forum.

One important question is whether raspberries are newly formed blood vessels or represent remodelling of the already existing vasculature. While the nomenclature has varied between different authors, the previously reported finding that I believe overlap the most with raspberries are arterioles running in parallel within a common perivascular space – termed ‘vascular bundles’ by Hassler (which he translated from Cerletti’s ‘Gefäßbündel’) [238, 239] (Fig. 13). Not all authors have specified where in the vascular tree that vascular bundles occur. Those who have, mainly appoint them to the branching of intermediate and deep penetrating arterioles [21, 32, 33]. Prior to progressing into individual perivascular spaces, the penetrating arteriole

sometimes give off multiple branches that run in parallel for some distance – a vascular bundle. The same pattern has been encountered within the branches originating from the penetrating arterioles [21]. Consequently, in relation to the cortical surface, vascular bundles could be running perpendicularly – as penetrating arterioles – and diagonally or parallelly – as branches. Depending on the angle by which a cortical tissue block is cut – which could vary somewhat between different parts of the block due to the three-dimensional folding of gyri and sulci – any of these vascular bundles could be identified as a raspberry in a histopathological tissue section. If vascular bundles run mainly at a particular angle – perpendicular, diagonal, or parallel to the cortical surface – the raspberry density may vary somewhat within a tissue section depending on the angle of the cut. However, this may not explain the full extent of the clusteriform distribution of raspberries that can be encountered occasionally (while many cases have a more diffuse distribution). Perhaps a 'cluster' of raspberries all originate from one or a few adjacent penetrating arterioles, all affected by some underlying process resulting in raspberry formation, either at the level of the penetrating arteriole, or at the level of the pial vessel that supplies adjacent penetrants.

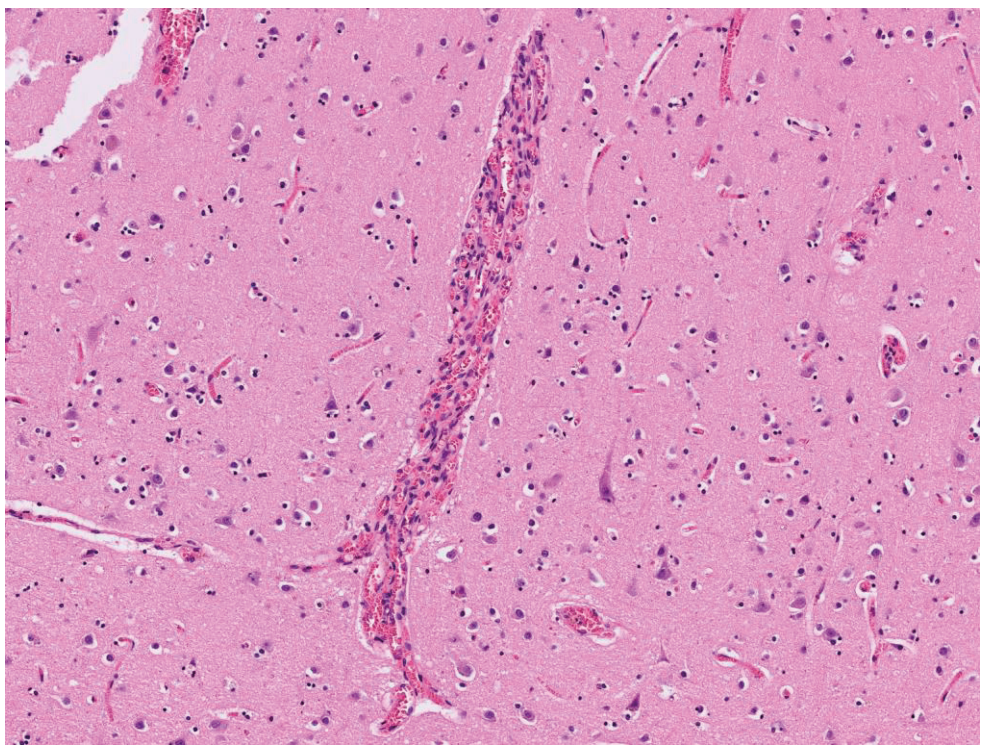

Fig. 13
Overview of a cortical vascular bundle with intertwining and branching vessels.

Another feature of vascular bundles reported by some authors is that they often intertwine like a rope (referred to as a separate entity, 'vascular wickerworks', by Hassler) [21, 32, 239]. However, intertwined or not, the authors who have examined these formations mainly concluded that they occurred due to an increase in arteriolar length, rather than atrophy of the surrounding parenchyma [239, 240]. If the surrounding parenchyma remained unchanged while the arterioles were elongated, the feature of multiple parallel arterioles within the same perivascular space could be expected to become more frequent, as would likely intertwining. If one supposes that such remodelling of the existing vasculature rather than de novo formation is the reason for variations in raspberry density, it could explain why raspberries occur mainly in certain brain regions – differences in branching patterns – and why they often occur in absence of any nearby focal lesions, as they may represent changes of chronical nature due to longstanding changes in the vascular environment.

As to why the arterioles would undergo remodelling – becoming elongated and intertwined – theories vary. In animal models of chronic hypoperfusion, arteriolar elongation and tortuosity has been observed [262], and it has been proposed that recurrent hypoxia could induce the same phenomenon in vascular bundles (and similar structures) [243]. However, since the increases in length and tortuosity were observed in collaterals [262], the affected vessels would have been exposed to increased perfusion [122]. Increased perfusion with altered shear stress is thought to be an important mechanism behind arteriogenesis, wherein smaller arterioles remodel into larger vessels [263]. According to one theory on the development of arterial/arteriolar tortuosity (a phenomenon that can be observed in arterioles in white matter and deep grey matter, as well as in arteries and arterioles in other parts of the body [122]), intraluminal pressure exceeding a certain threshold – for example, during increased perfusion – causes 'buckling' of the vessel [122]. The buckling results in uneven shear stress, leading to an equally uneven remodelling of the vessel, which would result in a permanent change in the vascular trajectory [122]. The threshold for when buckling occurs could be lowered by factors that weaken the vessel wall [122]. The theory has been applied mainly to explain arterial and arteriolar tortuosity affecting vessels of relatively larger calibre – I am not aware to what extent it can be translated to cortical arterioles, vascular bundles, and raspberries. For example, when Hassler quantified the abundance of vascular bundles in relation to arteriolar tortuosity (glomerular loop formations), he did not find a clear correlation between the two of them, perhaps indicating that the underlying mechanisms differ to some extent [240].

Another possibility is that raspberries are formed through de novo formation of blood vessels. Assuming that raspberries are mainly arterioles, this would require capillary sprouting – angiogenesis – followed by arteriogenesis. To my knowledge, whether arteriogenesis in adults occurs to some extent in vessels that were originally capillary sprouts formed in the adult individual – or solely by remodelling of pre-existing arterioles – is incompletely understood [264, 265].

As a final note, this section has been based on the assumption that raspberries are vascular bundles cut transversally – other potential three-dimensional correlates have not yet been excluded.

Clinicopathological characteristics of raspberries

In this section, the combined results of this thesis are discussed according to two major categories: raspberries in relation to vascular disease, and raspberries in relation to neurodegenerative disease. First, the association between raspberry density and age will be addressed in brief.

Age

Ageing is associated with alterations at the level of cells, tissues, and organs, affecting their function and structure, and increasing the risk of obtaining multiple diseases. Genetics and external factors may accelerate or decelerate this process, ultimately making it difficult to ascertain the exact influence of age on a studied phenomenon. In this thesis, we have attempted to examine to what extent age can be considered independently associated with raspberry density, by studying a wide age range while including multiple potential confounding factors in the analysis. Based on our results, it does indeed seem that the raspberry density is influenced by age. The results are in line with the findings of other authors on the synonymously defined 'multilumen vascular profiles' [246], and similar to many other vascular alterations, including arterial stiffening, arteriolar tortuosity, and arteriolosclerosis [95, 104, 126]. As such, raspberries can be considered a partially age-related phenomenon.

Vascular disease

However, like the age-dependent vascular alterations listed in the previous section, the abundance of raspberries can also be affected by factors beyond age. Provided that the statistically significant results from the papers – including the exploratory findings – are correct, how can they be interpreted?

Our results indicated positive associations between raspberries and cardiac hypertrophy, hypertension subgrouped for organ damage, cerebral atherosclerosis, and VaD, thus placing raspberries in a context of increased vascular burden. In this spectrum, VaD would represent an advanced stage of cerebrovascular disease, while variables that could occur in a context of less advanced cerebrovascular pathology include hypertension and cerebral atherosclerosis.

Cerebral atherosclerosis has been associated with microvascular alterations by some authors [149]; it may be difficult to conclude to what extent these entities share a causal relationship or simply represent vascular disease occurring at two different locations due to shared risk factors. Since our results indicate no more than a modest association between raspberry density and cerebral atherosclerosis, it is unlikely that cerebral atherosclerosis is an important cause of raspberry formation. More likely, both raspberries and cerebral atherosclerosis may be affected by similar factors, of which hypertension could represent one such common denominator.

There are many ways by which hypertension can affect the microvasculature. Increased intraluminal pressure may result in arterial buckling and tortuosity [122]. Endothelial dysfunction, microvascular rarefaction, and remodelling of the vascular wall have been proposed as potential contributors to reduced cerebral blood flow [55]. Perhaps a consequence of one of these processes could serve as a marker for the other processes. For example, even if the raspberries themselves were formed by other mechanisms than hypoperfusion (such as increased intra-luminal pressure), their presence could still be a marker indicating that the parenchyma was affected by hypoperfusion. This may be indirectly supported by the association between raspberries and VaD, given that some studies have demonstrated changes in protein levels suggestive of cortical hypoperfusion in this disease [170, 174]. While this is all speculation at this point, it would be interesting to examine further whether the raspberry density can be linked to similar signs of tissue hypoperfusion (see Outlook).

It is noteworthy that we did not find an association between raspberry density and arteriolosclerosis; they both occur in small vessels and appear to be associated with age, hypertension, and VaD. Excluding that such an association exists would likely require a semi-quantification of the arteriolosclerosis in relation to the raspberry density. This has not yet been performed by us; however, Ighodaro et al. semi-quantified arteriolosclerosis in relation to multilumen vascular profiles (synonymous to raspberries) and did not find them to be associated [246]. Possible interpretations could be that raspberries reflect a vascular alteration that begins to form prior to arteriolosclerosis in the progressive development of cerebrovascular disease [143], or that the mechanisms by which raspberries form are partially different from those that lead to arteriolosclerosis. A major difference between arteriolosclerosis and raspberries is that raspberries occur in the cortex rather than the white matter, while the opposite holds true for arteriolosclerosis [101]. However, a location where both raspberries and arteriolosclerosis occur is the deep grey matter, as raspberries are frequently encountered in the basal ganglia. Considering these regional variations, it could be interesting to examine whether cortical raspberries may be associated with arteriolosclerosis when grading arteriolosclerosis in white matter and deep grey matter separately. Due to the anatomy of the cerebral vasculature, the mechanisms leading to small vessel disease in white matter may differ from those in the deep grey matter [157]; if cortical

raspberries were to be associated with arteriolosclerosis in one of these regions but not the other, it might provide some notion of the mechanisms that contribute to their formation.

Whether excessive raspberry formation should be considered a form of small vessel disease would depend on their consequences. Speculating (boldly), one potential consequence could be altered drainage of interstitial fluid. Neuropathological features that might be indicative of altered perivascular drainage include widened perivascular spaces and CAA [25, 120]. Widened perivascular spaces are not commonly encountered in the cortex, perhaps due to regional variations in the anatomy of the pial sheaths enwrapping the vessels [20, 266]. My interpretation has been that while raspberries are frequently surrounded by an 'empty' space, this may represent an artefact rather than a pathologically widened perivascular space. However, it could be interesting to explore whether there is any variation between raspberries and single-barrel vessels in the cortex. Our results have not indicated an association with CAA; like arteriolosclerosis, semi-quantification of the CAA in relation to the raspberry density would be required to exclude this association.

Another possibility is that raspberries exert a negative impact on blood flow. If raspberries represent vascular bundles with increased arteriolar length and tortuosity, they may be associated with increased resistance and reduced blood flow, as estimated by other authors [122, 123, 240]. Perhaps such abnormal blood flow could affect the risk of microinfarcts [23]. A systematic examination of microinfarcts in relation to raspberries has not been performed – since microinfarcts are an important substrate for VaD [267], such an examination might teach us more about the potential clinical relevance of raspberries.

In addition to these potential future studies, it may be relevant to measure the vascular density in raspberry-rich tissue sections, since raspberries have been identified as more abundant in conditions generally associated with unchanged or reduced vascular density (age [95], VaD [150], hypertension [55]). This may be achieved by assessment of immunohistochemically labelled tissue sections; the immunohistochemical approach also has the advantage of allowing some characterisation of other microvascular properties, such as increased capillary diameters (potentially indicative of compensatory dilation due to hypoperfusion [150, 211]), and presence of string vessels (potentially indicative of capillary regression [121, 150]). Are raspberries associated with an increase in total vascular density, or is the total density decreased, along with increased capillary diameters or signs of capillary regression? The results of such a study could more information about the pathological significance of a finding of increased raspberry density.

Neurodegenerative disease

We found an increased raspberry density in FTLD but not in AD and LBD.

In AD, the raspberry density might even be decreased according to the results in Paper IV and the descriptive data in Paper V; however, this is contradicted by the results in Paper I. Paper IV has limitations regarding the capacity of examining the association between raspberries and neurodegenerative disease; this was not the primary aim of this paper. While a negative association between raspberry density and AD may not be biologically implausible – if raspberries are associated with hypertension, and mild hypertension late in life is protective against AD [180, 181] – I would not conclude that the raspberry density is decreased in AD, given the conflicting results and the limitations associated with some of the findings.

The high raspberry density in FTLD stands in some contrast to the other findings of this thesis. One may not exclude that the underlying mechanisms differ from those that mediate increased raspberry density within a setting of high vascular disease burden. That the results indicate an increased raspberry density in FTLD but not in AD or LBD is equally intriguing. All conditions have been associated with vascular and circulatory abnormalities in some form. Biochemically, findings suggestive of cortical hypoperfusion have been reported in AD (reduced MAG/PLP1 quotient [170]), LBD (reduced MAG/PLP1 quotient [210]), and FTLD-TDP type A with *GRN* mutation (HIF expression [227]). All conditions have further been associated with abnormal angiogenic signalling, mainly examined with regards to VEGF.

In AD, VEGF appears to be disproportionally increased in relation to the degree of hypoperfusion; in tandem, however, its availability may be reduced due to interactions between VEGF and amyloid beta [170, 187, 188]. The vascular density has been reported to be decreased in most studies [115].

In LBD, one study indicated that alpha-synuclein may inhibit VEGF expression and impair angiogenesis [210]. This and other studies demonstrated reduced capillary density in the cortex in LBD [150, 210, 211].

Similarly, a study by Gerrits et al. on the cortical gene expression in FTLD-TDP type A with *GRN* mutation reported reduced VEGF signalling; however, the expression VEGF-independent angiogenic factors was increased, as was the vascular density, even in areas that were unaffected by atrophy [227]. Increased vascular density and upregulation of angiogenic pathways in other diagnoses of the FTLD group have also been reported [224-226].

To my knowledge, it is not fully understood why expression of VEGF-independent angiogenic pathways resulting in increased vascular density would occur to a larger extent in (some types of) FTLD compared to other neurodegenerative diseases with reduced VEGF availability, or to what extent these potential differences are affected by variations in disease duration or age. From this perspective, it is notable that Paper V demonstrated an increased raspberry density in FTLD compared to LBD independent of age. It would be interesting to see whether the differences in raspberry density remain in the occipital cortex. Furthermore, clinical data on disease duration would be relevant.

If raspberries are indeed increased in the FTLD spectrum as a whole – or within part of the spectrum – it may add to recent studies on the pathophysiology of FTLD [224-227], such that vascular alterations may be a feature of FTLD that is not limited to specific mutations – even if the clinical significance of the raspberries themselves is currently unknown. Prior to making any such conclusions, however, our study population should be characterised in more detail. One important task will be to delineate the potential influence of brain atrophy on raspberry density. In our current results, we only found an inconclusive tendency towards a negative correlation between raspberry density and brain weight. However, as discussed under Methods, variations in brain weight may not be the optimal approach to examine the influence of atrophy in this setting. A rating of the atrophy within the examined tissue sections would likely be more informative, for example, by ranking the degree of cortical thinning [225]. An examination of the total vascular density and the presence of string vessels might provide further clues – if higher raspberry densities would correlate with findings of many string vessels in severely atrophic parenchyma, an influence of atrophy would be plausible, whereas the opposite results would point in another direction. Such investigations may further reveal whether the influence of atrophy varies between the different proteinopathies – for example, between FTLD-tau and FTLD-TDP.

Outlook

In addition to the methods available at our lab, we are currently planning for the application of other techniques and approaches in collaboration with fellow research groups, including three-dimensional visualisation of raspberries, transcriptomics, and animal models. A brief outlook is provided.

We have not yet performed a systematic three-dimensional visualisation of raspberries. As such, we do not know to what extent the three-dimensional correlates of raspberries are restricted to vascular bundles at the branching segments of arterioles. A study with the specific aim of defining the full three-dimensional spectrum of raspberries would be of value. One potential method of three-dimensional visualisation connotes software-assisted reconstruction of serially sectioned tissue blocks or biopsies (Fig. 14); this method has been applied by other authors on such structures as developing kidneys [268], pulmonary microvessels [269], and recently bone marrow [270]. Another technique by which three-dimensional visualisation can be achieved is microcomputed tomography (Fig. 15), previously applied in the study of microvascular pathology such as capillary dysplasia in the lung [271]. One advantage of both serial sections and microcomputed tomography is that they enable the use of formalin-fixed paraffin-embedded tissue, thus allowing the examination of archival brain tissue that can be selected based on histopathological or other characteristics.

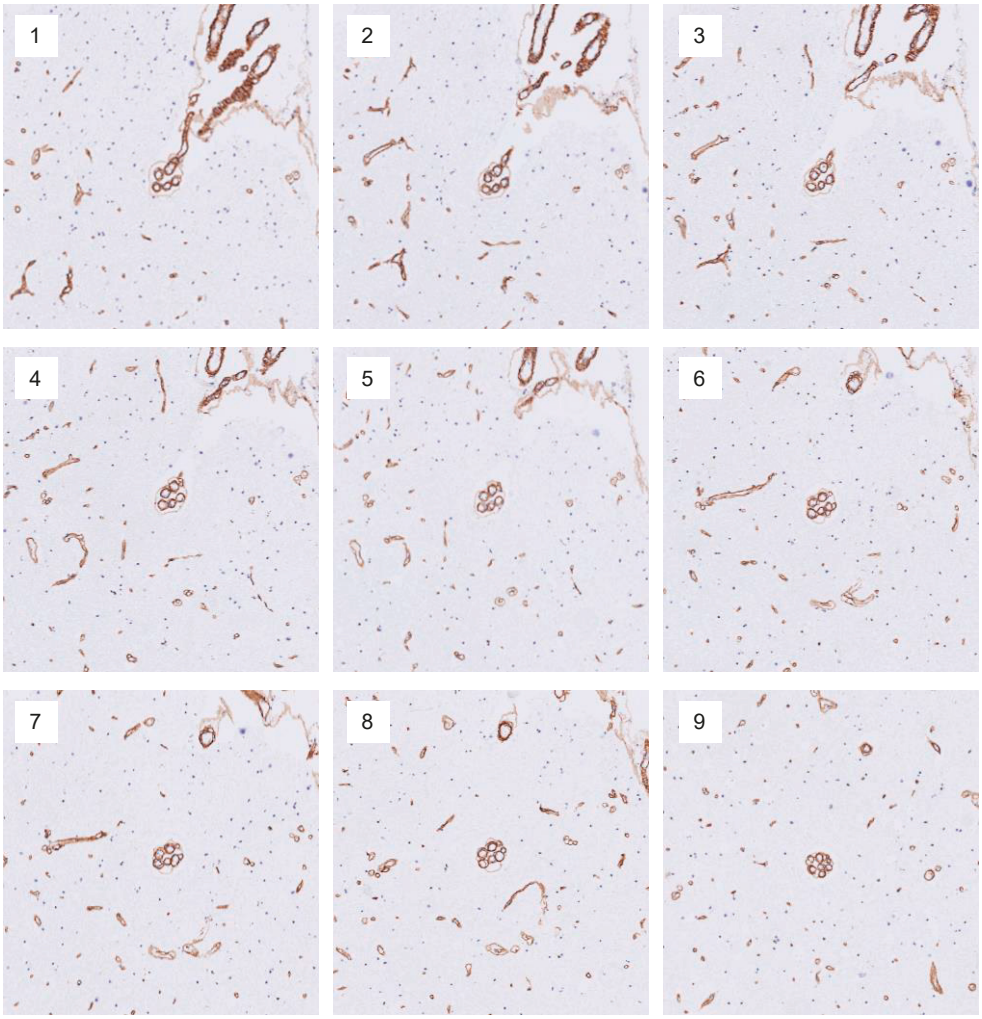

Fig. 14
Cortical tissue sections cut serially and immunohistochemically labelled for collagen 4.

Single cell transcriptomics provide information on the gene expression in different cell populations. In the context of raspberries, this technique could provide data beyond the histopathology regarding the composition and state of the tissue, thus improving our understanding of what a finding of abundant raspberries within a tissue section might represent. One challenge lies in correlating the gene expression with the raspberry density; the raspberry density of the tissue being freshly frozen for transcriptomics may need to be estimated from an adjacent tissue block that is being formalin-fixed and paraffin-embedded.

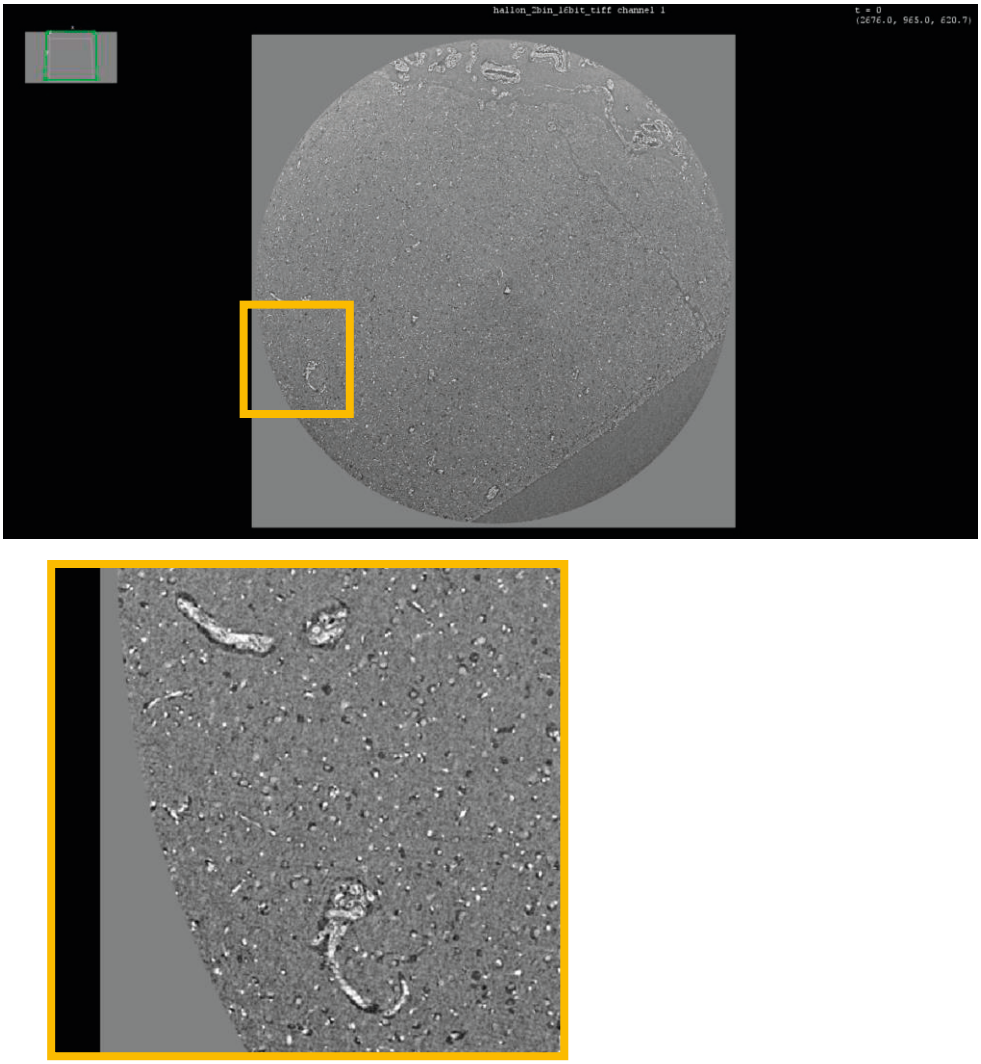

Fig. 15
Identification of potential raspberry correlates by microcomputed tomography of cortical formalin-fixed paraffin-embedded tissue blocks. Courtesy of Martin Bech.

Ultimately, the question of whether cortical raspberries form because of hypoperfusion may require support from animal models of chronic hypoperfusion to prove or disprove. Capable of inducing features similar to human small vessel disease and white matter disease, animal models of chronic hypoperfusion may provide clues on whether raspberries and white matter arteriolosclerosis form by similar mechanisms. To my knowledge, only one previous study has looked at raspberry-like formations in animals – Hassler identified intermediate forms of the

studied vascular entities (glomerular loop formations/arteriolar tortuosity, vascular bundles, and vascular wickerworks) in dogs and horses but not in guinea pigs; the examined animals exhibited signs of old age and had not undergone any interventions [239]. The potential influence of differences in vascular anatomy between humans and animals should be considered.

Conclusion

I wrote before about a jungle of small vessel alterations, but perhaps it can be likened to a garden as well. In the garden, various scientists take an interest in particular aspects of the different plants, be it the branches (arteries), leaves (capillaries), the pot that waters the plant (the heart), or – as in this thesis – the raspberries, that grow on some of its minor twigs.

Putting it more frankly, this study has examined a microvascular, neuropathological feature termed 'raspberry', a finding frequently encountered in the cerebral cortex and deep grey matter but rarely in the white matter. By measuring the raspberry density in the neocortex, the clinicopathological characteristics of inter-individual variations in raspberry density have been outlined.

The raspberry density is positively associated with increasing age, also when including multiple potential confounding factors in the analysis. The major increase may occur in the 7th or 8th decade of life, although the average age of onset may be lower. As such, raspberries can be considered a partially age-related feature.

However, positive associations between raspberry density and cerebral atherosclerosis, hypertension subgrouped for organ damage (exploratory), and vascular dementia indicate that the raspberry density is further increased in a context of higher vascular disease burden.

Regarding neurodegenerative diseases, the main finding of this thesis is an increased raspberry density in frontotemporal lobar degeneration (FTLD) but not in Lewy body disease or Alzheimer's disease. Recent studies by other authors have indicated neurovascular dysfunction in the pathogenesis within part of the FTLD spectrum. The significance of the increased raspberry density in FTLD is unknown, and the immediate future research should focus on determining whether the influence of brain atrophy on raspberry density varies within this heterogeneous group of diseases.

Important steps towards improving our understanding of raspberries include a more detailed examination of raspberries in relation to total vascular density, regional arteriolosclerosis, cerebral amyloid angiopathy, and cortical microinfarcts, and an examination of the gene expression in raspberry-rich brain tissue.

A potential correlate of raspberries connotes vascular bundles that form at the branching segments of cortical arterioles – a systematic examination of the three-dimensional spectrum of raspberries would be of value to strengthen or refute this view.

In addition to our original hypothesis on hypoperfusion as the driving force behind raspberry formation, theories on the formation of arteriolar tortuosity may be considered. Whether raspberries form in a context of chronic hypoperfusion may be tested in animal models; such studies may further entail whether raspberries form by similar mechanisms as white matter arteriolosclerosis.

Fully establishing the pathogenesis, consequences, and clinical relevance of raspberries has not yet been achieved, but the current thesis presents an initial attempt to narrow down the clinicopathological characteristics beyond age.

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