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Mixed dementia in Alzheimer's disease associated with cerebrovascular disease: environmental risk factors, clinical and radiological features, and the role of fluid biomarkers

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ABBREVIATIONS

ABI: ankle-brachial index

AF: atrial fibrillation

AD: Alzheimer's disease

ADAD: Autosomal dominant Alzheimer's disease

AD-CVD: mixed AD and cerebrovascular disease dementia

AF: atrial fibrillation

AP: air pollution

APs: Air pollutants

ApoE: apolipoprotein E

ApoEX: ApoE εX

ARIA: amyloid-related imaging abnormalities

AQ4: aquaporin 4

AUC: area under the curve

BBB: blood-brain barrier

BM: Biomarker

BNT: Boston Naming Test

cf_PWV: carotid-femoral pulse wave velocity

CAA: cerebral amyloid angiopathy

CBI: covert brain infarcts

cCVD: covert cerebrovascular disease

CVD: cerebrovascular disease

CHA: chronic hydrocephalus in adults

CMB: Cerebral microbleed

cSS: cortical superficial siderosis

CSF: cerebrospinal fluid

CNS: central nervous system

CSO: centrum semiovale

CT: Computed Tomography

CV: coefficient of variation

CVD: cerebrovascular disease

CVRF: cardiovascular risk factors

DALY: disability-adjusted life years

DBP: Dyastolic blood pressure

DMT: Disease-modifying treatments

DSAD: Down Syndrome Alzheimer's disease

DWI: diffusion-weighted imaging

ELISA: Enzyme-Linked Immunosorbent Assay

FDR: false discovery rate

FLAIR: Fluid-Attenuated Inversion Recovery

FTD: Frontotemporal Dementia

GCA: General Cerebral Atrophy

GDS: (Reisdeberg's) Global Deterioration Scale

GFAP: Glial Fibrillary Acidic Protein

HIC: high- income countries

I**CH**: intraparenchymal haemorrhage

IHD: ischaemic heart disease

IQR: interquartile range

LATE: Limbic-predominant Age-related TDP-43 Encephalopathy

LBD: Lewy's body dementia

LOAD: late onset Alzheimer's disease

LMIC: Low- and middle- income countries

LUR: Land-use regression models

MCI: mild cognitive impairment

MMSE: MiniMental Scale Examination

MRI: Magnetic Resonance Imaging

MTA: medial temporal atrophy

NA: normal (cognitive) aging

NfL: neurofilament light chain

NPS: neuropsychological

NOx: nitric oxides

NO2: nitrogen dioxide

OR: odds ratio

PD: Parkinson's disease

PET: positron emission tomography

PM: particulate matter

PV: periventricular

PVS: perivascular spaces

RCT: randomized clinical trials

ROC: receiver operating characteristic

SAH: subarachnoid haemorrhage

SC: subcortical

SD: standard deviation

SIMOA: Single Molecule Array

sTREM2: soluble fraction of the Triggering Receptor Expressed on Myeloid cells 2

SPECT: Single photon emission computed tomography

svCVD: small vessel cerebrovascular disease

TIA: transient ischemic attack

TBI: traumatic brain injury

TMT: Trail Making Test

VCI: vascular cognitive impairment

VaD: vascular dementia

VIF: variable inflating factor

WMHs: white matter hyperintensities

ABSTRACT

Background:

Diagnostic and therapeutic advances in Alzheimer's disease (AD) have underscored the importance of better understanding mixed Alzheimer's and cerebrovascular disease (AD-CVD), including cerebral amyloid angiopathy (CAA). A deeper knowledge of this prevalent association could provide valuable insights into the role of cerebrovascular disease (CVD) in AD and, more broadly, in other neurodegenerative conditions, while also informing the development of preventive and therapeutic interventions.

Another understudied relationship is that between pollution (AP) -a shared risk factor for both AD an CVD- and cognition and covert CVD (cCVD), defined as CVD that develops and progresses without evident symptoms or acute clinical events. Advancing our understanding of this relationship may likewise contribute to the design of effective preventive policies.

Methods:

Two separate studies were conducted to address these aims.

Study One:

We aimed to comprehensively characterize patients with AD-CVD by integrating clinical assessments, neuropsychological (NPS) testing, and imaging and biofluid biomarkers (BMs), and by comparing them with patients with AD without significant CVD. We hypothesized that this multimodal evaluation would allow us to differentiate between the two groups and predict group membership.

We prospectively recruited individuals with mild AD confirmed by cerebrospinal (CSF) BMs, either with (AD-CVD) or without significant CVD. All participants underwent clinical

and NPS evaluations, cerebral magnetic resonance imaging, and biofluid (plasma and CSF) biomarker assessments. Group classification was based on a combination of established diagnostic criteria. A control group with plasma BM data was also included. We performed a sensitivity analysis excluding individuals classified as AD-CVD solely based on extensive white matter hyperintensities (WMHs).

Study Two

We aimed to study the association between AP and cCVD and cognition. We hypothesized that greater exposition would result in poorer cognition and a greater cCVD burden.

We estimated exposure to six air pollutants (APs) -NO2, NO_x, PM_{10}, $PM_{2.5}$, PM_{coarse} and $PM_{2.5absorbance}$ - for each participant in a cohort of hypertensive individuals without a prior history of stroke or dementia using a land-use-regression model. Participants were drawn from the ISSYS (Investigating Silent Strokes in hYpertensives Study) cohort, a population-based prospective study conducted in Barcelona between 2010 and 2016. Cognitive function and cCVD were assessed through cognitive evaluations and cerebral MRIs both at baseline and after four years of follow-up.

We analysed associations between estimated AP exposure and both cCVD and cognitive outcomes, both cross-sectionally and over time.

Results:

Study One:

The only significant difference between groups was a faster functional decline in the AD-CVD group, although this was not observed in the sensitivity analysis. In this analysis, plasma t-tau levels were higher in the pure AD group.

The area under the curve (AUC) for blood ucMGP in distinguishing AD from AD-CVD was 0.706. In the sensitivity analysis, additional BMs demonstrated an AUC greater than 0.7, including t-tau (0.74), sTREM2 (0.71), and ucMGP (0.72). sTREM2 was associated with non-specific CVD BMs, and ucGMP with lobar CMBs. Extensive WMHs were correlated with more severe medial temporal atrophy. About one third of participants fulfilled criteria for either possible or probable CAA. No BM correlated with the CAA burden.

Study Two:

Exposure to $PM_{2.5}$ was associated with higher odds of covert brain infarcts (odds ratio (OR) 2.21; confidence interval (CI) 95% 1.06-4.60). $PM_{2.5\ absorbance}$ exposure was linked to greater odds of extensive subcortical WMHs (OR 1.72; CI95% 1.13-2.60). Exposure to NO_2 was associated with extensive subcortical WMHs (OR 1.66; CI95% 1.17-2.35), extensive periventricular WMHs (OR 1.96; CI95% 1.10-3.50), and marked subcortical WMH progression (OR 1.40 CI95% 1.05-1.90). NO_x exposure was associated with incident cerebral microbleeds (OR 1.36 CI95% 1.04-1.79). Both NOx and NO_2 were associated with a higher combined progression score. There was no association between AP and cognition.

Conclusions:

AD and AD-CVD exist along a shared continuum, in which CAA is frequent. ucGMP, sTREM2, and t-tau are promising BMs within such continuum for distinguishing AD and AD-CVD. Extensive WMHs indicate a poorer prognosis.

Exposure to several APs predicts the presence and accumulation of cCVD and may represent a modifialbe risk factor for cCVD in individuals already predisposed to it. Its impact on cognitive impairment remains to be determined.

Introduction

Overview and rationale for the studies

Neurological diseases are the major cause of disability-adjusted life years (DALY) worldwide (1). Specifically, both cerebrovascular disease (CVD) - including stroke- and cognitive impairment rank among the primary causes of death and disability at the global level.

Cognitive impairment is an umbrella term used to describe a group of symptoms that affect a person's cognitive abilities, such as their memory and language. When cognitive impairment interferes with the individual's normal functioning, it is referred to as dementia (2). In contrast, mild cognitive impairment (MCI) refers to a condition in which an individual has an objectively determined cognitive impairment but remains functional and able to carry out most daily activities independently (3). Although the age-specific incidence and prevalence of dementia have decreased over time, particularly in high-income countries, its global prevalence is expected to rise globally in the coming years due to population aging (4). A significant portion of this increase will occur in low- and middle-income countries (LMICs), where the majority of the population with dementia is projected to reside in the future (5). Since the clinical, economic and social impact of dementia is huge, any action taken to reduce its incidence would have enormous benefits at the population level.

Overall, the most common cause of dementia is Alzheimer's disease (AD) (6). This neurodegenerative condition does not only impact affected individuals' health but their caregivers' (7). Consequently, AD is by large the most extensively studied condition in the field of dementia.

The use of biological markers or biomarkers (BMs) - which are measurable indicators of a biological condition or process- has facilitated a shift from a clinical-pathological

framework to a biological definition of neurodegenerative diseases (8,9). Such a shift has enabled both diagnostic and therapeutic advances, overcoming some of the limitations of diagnosis based solely on clinical criteria.

Notably, it allows to diagnose the disease with high certainty early in their time course, including preclinical stages. In turn, this enables to implement secondary prevention strategies or disease-modifying treatments (DMTs) at the earliest possible moment, potentially altering the trajectory of the disease. Beyond enabling a more accurate and timely diagnosis of AD, BMs can also monitor disease progression. The availability of tools for making a biological diagnosis has significantly increased in recent years, particularly for AD. This is well-illustrated by the incorporation of blood AD BMs into clinical practice (10). In the therapeutic landscape, lecanemab and donanemab have emerged as the first disease-modifying treatments (DMTs) for AD (11,12). Leqembi (lecanemab) has already been approved by the US Food and Drug Administration, and, more recently, by the European Medicines Agency (EMA) (13,14). Therefore, these advances present a critical opportunity to reduce the incidence and impact of dementia (15).

Although this shift has been led by the one made in AD, it is also taking place in most of the other most prevalent neurodegenerative conditions, such as frontotemporal dementia (FTD) spectrum disorders and synucleinopathies (8,9).

Neuropathological studies and, more recently, BMs research indicate that AD pathology is rarely isolated. Instead, AD is frequently accompanied by other pathologies to varying degrees, which can influence both clinical presentation and BM profiles.

This includes Alzheimer disease associated with cerebrovascular disease (AD-CVD), the prevalence of which has been reported to range from 5% to 35% in several clinical-pathological cohort studies (16,17). This is consistent with AD-CVD being one of the commonest causes of dementia, and with CVD being the most prevalent co-pathology

in both genetically determined and sporadic AD. Several environmental and genetic risk factors and shared pathophysiological pathways and processes drive this association, including APOE genotype (18–20). ApoE4 favours the development of both AD and CVD, and, specifically, of cerebral amyloid angiopathy (CAA) (21,22). CAA consists of beta-amyloid deposition in leptomeningeal and small cerebral blood vessels, it is a common cause of Intracerebral haemorrhage (ICH) and it is frequently found in AD patients (23).

However, BMs and therapeutic strategies for non-AD pathologies with the potential to impair cognition are still relatively underdeveloped in comparison to AD, including AD-CVD (17). The heterogeneity and complexity in the mechanisms and clinical manifestations underlying CVD, may have impeded more thorough investigation and a clearer and more widely accepted definition of AD-CVD.

Therefore, a high proportion of people living with cognitive impairment is not well-addressed by current available diagnostic and therapeutic evidence, which is mostly driven by- and focused in- “isolated” AD rather than on “mixed” cases (co-pathology scenarios). As a mode of example, AD-CVD cases have been systematically excluded from randomized clinical trials (RCTs) for AD, including those evaluating lecanemab and donanemab. Notably, in these RCTs, the majority of serious adverse events – particularly amyloid-related imaging abnormalities (ARIA) (which include cerebral oedema and/or haemorrhages)- occurred in individuals carrying the ApoE4 allele (11–14).

Therefore, there is a pressing need to better understand the role of CVD both at the pathophysiological and clinical level in dementia populations, beginning with AD, including its interactions with neurodegenerative pathologies and the underlying causes and risk factors. In other words, it is critical to enhance research efforts to better characterize the clinical and paraclinical (BMs) profiles of AD-CVD. Deeper investigation into AD-CVD could enhance our understanding of AD, VCI, CVD and their overlap – including the role of CAA in AD-CVD and its association with ARIAs- and, ultimately,

contribute to the development of more targeted interventions both at the therapeutic and preventive level.

With regards to the latter, preventing exposure to some ubiquitous environmental hazards -factors that may adversely affect health (24)- has the potential of bearing health benefits to wide sectors of the world's populations. Air pollution (AP) is a good example of it, as 90% of the world's population is exposed to it (25), and it has been associated with both CVD and AD (26). Moreover, because the primary source of AP is the combustion of greenhouse gases– which are also the main drivers of climate change- reducing AP exposure could generate substantial co-benefits, including functioning as a mitigation strategy for climate change.

This introduction aims to provide an overview of how AD, CVD, VCI, and their overlap (AD-CVD) have been conceptualized and defined, highlighting the inherent complexity of these conditions.

The section will begin by introducing the concepts of CVD and VCI, along with their most common causes, followed by a discussion on AD-CVD. BMs for AD, CVD, VCI and AD-CVD will then be reviewed, including a summary of current evidence on their use in AD-CVD and their potential for advancing the understanding of the relationship between AD and CVD. Subsequently, potential mechanisms underlying this association will be explored, as these may offer insight into the clinical presentation, disease progression, and therapeutic targets. These mechanisms will include AP as a shared environmental risk factor for both AD and CVD. Finally, a brief discussion on prevention strategies will be provided.

Cerebrovascular disease

CVD is a broad term that refers to all diseases in which the blood vessels of the brain are primarily involved (27). Stroke and transient ischaemic attack (TIA) -acute symptoms due to the occlusion (ischaemic stroke) or rupture (haemorrhagic stroke) of a blood

vessel are the most well-known clinical manifestation of CVD. Stroke episodes and up to 30-50% of TIA episodes can lead to brain infarctions, which are usually evaluable with neuroimaging techniques (27). However, CVD can occur and progress without any acute episode, leading to a progressive deterioration of brain functions such as cognition and gait.

cCVD

"Covert cerebrovascular disease" (cCVD) is the term used to refer to the cases in which cerebrovascular lesions occur and progress silently- that is- without provoking any apparent symptom nor acute episode, such as stroke or TIA (28).

In the absence of a temporal relationship with a stroke, it is often difficult to establish the clinical significance of CVD lesions with regards to cognition. It is widely accepted that brain infarcts affecting certain regions can, on their own, cause significant cognitive deficits, what is sometimes referred to as "strategic infarcts". For instance, subcortical brain infarcts in the internal capsule, the thalamus, the caudate nucleus and several parts of the corpus callosum can all lead to significant cognitive deficits (29). Likely, existing evidence suggests that it is the volume in certain strategic locations rather than the entire burden of white matter abnormalities, which are visible in MRI as WMHs, which is most associated with cognitive impairment (29).

However, lesions other than these can also cause clinically significant cognitive impairment. In this sense, initiatives such as the Vascular Cognitive Impairment Neuropathology Guidelines (VCING) have been valuable. The VCING established which types of vascular lesions are the most associated with VCI: large (>10 mm) subcortical cerebral infarcts, moderate or severe occipital leptomeningeal CAA, and moderate or severe occipital white matter arteriosclerosis (30). From a radiological standpoint, the Vascular Cognitive Disorders Study Group of the International Society for Vascular

Behavioural and Cognitive Disorders (VASCOG) criteria (31) established a consensus on what can be considered a “significant” CVD.

Causes of CVD

Overall, the most frequent causes of CVD are large vessel and small vessel disease and/or cardiac conditions that increase the risk of cardioembolism. The most common cause of both large and small vessel disease is atherosclerosis -an inflammatory process of the vascular wall characterized by plaque formation, which can lead to vessel stenosis, thrombosis, or atheroembolism. Traditional cardiovascular risk factors (CVRFs), such as hypertension, hypercholesterolemia, diabetes mellitus, and smoking, are the primary etiological contributors. The most common cardiac condition leading to cardioembolism is atrial fibrillation (AF); however, other conditions, such as myocardial infarction and valvular heart diseases, can also contribute (27).

The following section will focus on small vessel cerebrovascular disease (svCVD), as it represents the most common cause of vascular cognitive impairment (VCI) (32)

Small vessel cerebrovascular disease

svCVD is a very broad concept that includes different processes that lead to pathological changes in the arterioles, venules and capillaries of the brain. Thus, the pathophysiological and clinical heterogeneity is huge, exemplifying well the complexity inherent within the CVD and VCI concepts. Several definitions- clinical, radiologic, pathologic- and classifications (TOAST, CSS, ASCOD, STRIVE) of svCVD have been developed.

svCVD increases the risk of stroke, dementia, and mortality, as illustrated in large population-based cohorts (33).

The most common pathologies leading to svCVD are atherosclerosis, arteriolosclerosis and CAA, although other causes are possible, such as inherited aetiologies (CADASIL, MELAS, Fabry), inflammatory disorders (systemic vasculitis, post-infectious, primary angiitis of the CNS) or venous collagenosis (34). Identifying the underlying pathology, for which the radiological features are often helpful, may be useful to establish the most appropriate management. The following section will focus on CAA, as its understanding is critical to the topic at stake for several reasons, as further specified below.

Cerebral amyloid angiopathy

CAA is characterised by amyloid-β deposits in the walls of leptomeningeal and cortical blood vessels (21). These deposits disrupt the structure and function of the vessel and impair their autoregulation. This leads to chronic hypoperfusion (and widespread ischaemic changes), BBB breakdown, neuroinflammation, and increased vessel fragility, which may rupture and intraparenchymal haemorrhage (ICH) or subarachnoid haemorrhage (SAH) (or its chronic form: cortical superficial siderosis (cSS)) or smaller haemorrhagic lesions such as cerebral microbleeds (CMBs). This progressive condition represents a neurodegenerative form of CVD.

It has substantial clinical implications, as it is the leading cause of ICH in the elderly and, regardless of ICH, it can also contribute to gait disturbances and cognitive impairment (23). It can also cause transient neurological focal symptoms -referred to as “amyloid spells”- which are thought to be related to cSS. In some cases, it manifests more severely in a subacute or acute inflammatory form, known as inflammatory CAA (35).

While most CAA cases are sporadic, familial forms associated with specific genetic mutations have been described. Iatrogenic cases have also been reported. Iatrogenic and genetic causes should be considered in patients presenting with early-onset CAA (35).

There is a significant overlap between CAA, in which the amyloid (mostly Aβ-40) deposition occurs in the blood vessels, and AD, in which the amyloid (mostly Aβ-42) deposition occurs in the brain parenchyma. For instance, in a 2023 study 26.7% individuals with AD met criteria for probable CAA and 42.7% met criteria for possible CAA (36). Neuropathologically, CAA has been found in more than 85% of cases with sporadic AD (37). This close association between the two conditions -which has been even referred to as a “cerebrovascular amyloidosis continuum” is probably due to the shared amyloid-related pathophysiological mechanisms, which may interact with each other in their development and evolution (38). For instance, vascular dysfunction caused by CAA reduces Aβ clearance, which further accumulates in vessels and/or cerebral parenchyma. There are factors that favour vascular Aβ deposition over parenchymal deposition, such as the longitude of the Aβ peptides (39).

The most used criteria to diagnose the condition is a combination of clinical symptoms and radiological and pathological signs, enshrined in the Boston criteria, which had its second version published in 2022 (36). The definitive diagnosis requires a postmortem examination demonstrating severe CAA with vasculopathy, but a “probable” diagnosis can be made in vivo solely with the clinical presentation and an MRI. The main differences between the first and the second version of the Boston criteria for the diagnosis of CAA are summarised in Table 1. In short, the second version considers, in addition to ICH, cognitive impairment and transient neurological symptoms as clinical presentations of the disease. Further, characteristic svCVD non-haemorrhagic lesions, such as WMHs with a multisport pattern and enlarged perivascular spaces (PVS) in the CSO, are now included into the diagnostic criteria. Since they tend to occur earlier in the

timeline course of the disease than haemorrhagic lesions,, they facilitate the prompt diagnosis of the disease by increasing the sensitivity of these diagnostic criteria in the early stages of the disease up to 91% for the possible category and up to 74.5% for the probable category (40). Importantly, the presence of at least one lobar haemorrhagic lesion -CMBs, ICH or cSS -is still needed for the probable category. However, it has been argued that, in the absence of ICH, the second version of the Boston criteria is not as accurate as the previous version (Boston criteria version 1.5.) (36).

Although not routinely used in the clinical setting, BMs related to amyloid pathology -further explained below- are usually abnormal in CAA, like in AD. However, some differences have been described, such as lower levels of CSF-Aβ1-40 in CAA compared to controls and AD patients, and normal levels of phospho-tau (41,42). However, differentiating with high certainty between CAA, AD, and CAA associated with AD based solely on the amyloidosis BMs that are currently available in the clinical practice is fraught with difficulty (21).

Table 1. Differences Between Versions 1.5 and 2.0 of the Boston Criteria

	Boston Criteria v1.5 (2010) (43)	**Boston criteria v2.0 (2022)** (40)
Commonalities	Individuals of ≥55 years with a suggestive clinical picture AND at least one radiological BM of CAA for the "possible category" and at least two for the "probable category". Both require the absence of a more plausible explanation for the findings.	
Main differences	The clinical picture is restricted to an episode of lobar ICH. It only considers haemorrhagic radiological BMs of CAA: ICH, CMBs or cSS.	The clinical picture is broadened to include ICH and/or cognitive impairment and/or transient cognitive symptoms ("amyloid spells"). Non-haemorrhagic BMs of svCVD are also included, are also included; however, for classification under the "probable" category, the presence of at least one haemorrhagic marker is required. The svCVD non-haemorrhagic BMs which are considered are: • WMHs with a multispot pattern (>10 in the subcortical white matter of both hemispheres). • >20 EPS in the CSO (of one hemisphere).

Currently, there are no curative treatments available for this condition, and management mostly consists in secondary prevention of ICH through strict blood pressure control and other measures.

Several additional factors contribute to CAA relevance when addressing AD, CVD or AD-CVD.

First, understanding the relationship between this neurodegenerative condition affecting primarily cerebral blood vessels and AD is intrinsically valuable. Further, CAA may be one of the mechanisms underlying the frequent association between AD and CVD.

Second, its prevalence is notable, and tools to assess it in vivo are now available. Although estimates vary across studies - likely due to differences in populations and diagnostic criteria - a systematic review and meta-analysis reported a prevalence of moderate to severe CAA ranging from 22% to 48% in AD populations and from 7% to 23% in the general population, with higher rates observed in neuropathological studies and lower rates using MRI criteria (43). The discrepancy between imaging and neuropathological methods for CAA assessment suggests that imaging is sufficiently sensitive only to detect the most severe cases. Notably, this meta-analysis was conducted before the publication of the second version of the Boston criteria for the diagnosis for CAA diagnosis (36,43).

Finally, its presence is closely linked to the development of ARIA, the most serious adverse event related to the administration of the anti-amyloid DMTs such as lecanemab (13).

Vascular cognitive impairment

Similarly to AD-CVD, one of the main reasons for the poor understanding of VCI is its complexity, reflected in the fact that no single widely accepted definition of VCI exists so far, and many definitions and diagnostic criteria have been developed instead (44). In short, VCI is the term used to describe cognitive impairment resulting from impaired cerebral blood flow or, in other words, the full range of cognitive disorders associated with CVD, regardless of the specific mechanisms involved (45).

As mentioned in the previous section, several mechanisms can lead to CVD. Likely, several mechanisms can lead to VCI. In turn, several aetiologic entities and risk factors can be involved in such processes, including traditional CVRF, cardiopathies, genetic conditions, and CAA.

Traditionally, it was mostly understood as cognitive impairment developing after a stroke and/or within the context of evidence of significant CVD in the neuroimaging. However, the VCI concept has evolved over the years since its first description by Hachinski in 1974 and now includes a much larger spectrum, including different grades of severity and a wide range of different pathophysiologic mechanisms- regardless of stroke history or symptoms- and allowing for the possibility of co-pathology, which occurs in about half of the VCI cases (46). These facts are emphasized in the VICCCS (Vascular Impairment of Cognition Classification Consensus Study) diagnostic criteria (47), which acknowledge the possibility of co-pathology and state that a history of stroke or a temporal relationship between clinical onset or worsening with a stroke episode is not necessary to establish a VCI diagnosis.

As stated in the corresponding section below (neuroimaging for VCI and svCVD), the svCVD picture of an individual is dynamic. In line with this, VCI might develop beyond the first 6 months after a stroke, a phenomenon which has been enshrined in the term “delayed onset VCI”. This phenomenon occurs in up to 10% of post-stroke VCI. The occurrence of this phenomenon in the absence of AD - as demonstrated by negative amyloid BMs- has been demonstrated (48). Several radiological features, such as the combination of confluent WMH (grade 3/3 of the ARWMC scale (49) and multiple lacunes (3 or more) after a stroke, have shown to increase the risk of delayed-onset post-stroke dementia (48). Likely, WMHs volumes at 1 year time after a stroke were seen to be associated more strongly with poor cognition than age or NIHSS score (50). VCI after a lacunar stroke might be therefore more related to the underlying svCVD than to the stroke itself, which may represent only the tip of the iceberg.

The potential of VCI to evolve progressively is consistent with the nonspecific nature of its clinical and neuropsychological (NPS) features, which can resemble those of neurodegenerative conditions. For instance, despite current consensus statements maintain that more “cortical” cognitive functions such as language and memory, are relatively preserved in svCVD-related VCI, research indicates that cognitive impairments in svCVD extend beyond executive functions and that can be found across several cognitive domains (32). Most of the cognitive deficits associated with svCVD likely stem from changes in white matter networks, particularly those within fronto-subcortical pathways.

The most appropriate complementary test to evaluate VCI is cerebral MRI. Neuroimaging in VCI will be addressed in the corresponding section.

Co-pathology: the AD-CVD case and its representation in current diagnostic criteria

As stated above, co-pathology -the coexistence of multiple pathologies in a single individual- is the rule rather than the exception in cognitively impaired populations, particularly with increasing age; and CVD is among the most common co-pathologies of AD (51).

However, as summarised in Table 2, many diagnostic criteria for AD or VCI do not even consider the possibility of co-pathology or underestimate its importance. Among those which do, the terminology is highly variable. Furthermore, there is no accepted consensus on the neuropathological definition of AD-CVD (51).

However, given the growing recognition of the importance of co-pathology, this is now reflected in several of the latest diagnostic criteria for AD and/or VCI. Notable examples include the VICCCS criteria (52), the International Working Group (IWG)-2 criteria (53),

and the US National Institute of Aging and the Alzheimer's Association (NIA-AA) 2024 Alzheimer's Association Revised Criteria and Framework for the Diagnosis and Staging of Alzheimer's disease (54). The NIA-AA 2024 criteria, in particular, incorporate a "V" (vascular) and an "S" (synucleinopathy) component into the classical ATN framework (Amyloid, Tau, Neurodegeneration), thereby expanding the construct to more comprehensively capture mixed pathologies (54).

Table 2. Summary of diagnostic criteria for AD, VCI and/or AD-CVD

Name of the criteria	Condition primarily addressed	Mode of assessment	Last version (year)	Commentaries on the criteria
NINCDS-ADRDA(192) /NINDS-AIREN (243)	AD, VaD	Clinical	1984 (AD), 1993 (VaD)	In the diagnostic criteria for AD, CVD is considered only as a contributing factor to AD pathology. Conversely, in the criteria for VaD, AD is mentioned just as a differential diagnosis.
CERAD(244,245)	AD	AP (mostly), clinical	1985	Vascular lesions are not considered.
Braak's(246)	AD	AP	1991	Vascular lesions are not considered.
VICCCS(55)	AD_CVD	AP	2007	Among the first efforts to describe the neuropathological profile of AD_CVD.
AHA/ASA(113)	VCI	Clinical	2011	The possibility of an associated neurodegenerative disease is considered in the "possible" category, but not in the "probable" one.
DSM-V(247)	AD, VCI	Clinical	2013	The presence of another possible aetiology of the symptoms is included as an exclusion criterion.
IWG 2 criteria(53)	AD, AD_CVD	Clinical, BMs	2014	Considers specific clinical and imaging criteria for "mixed AD", either associated with CVD or LBD.
VASCOG(31)	VCI	Clinical, radiological	2014	Developed by experts on the topic to state what a "significant CVD" is from a clinical and radiological point of view.
VICCCS(47,52)	VCI	Clinical	2016	International consensus (using the Delphi method) on the terminology and diagnosis of VCI. Includes "mixed dementia" as one of the four main types of VCI, and also recognizes potential co-pathology with neurodegenerative diseases across all other categories: post-stroke dementia, subcortical ischemic vascular dementia, and multi-infarct (cortical) dementia.
VCING(30)	VCI	AP	2016	Agreement among neuropathologists (Delphi method) on a scoring criterion,followed by itsempirical testing. Cases with substantial AD pathology (defined by Braak tangle stage>III) were excluded from the study.
NIA-AA(54)	AD, (AD_CVD)	Clinical, BMs	2024	Biological approach. AD is defined biologically using BMs. The framework incorporates a "V" to indicate CVD and an "S" for synucleinopathies.
IWG 3 criteria (87)	AD	Clinical> BMs	2024	More comprehensive approach. Additional factors are necessary to define ADbeyond positive AD BMs.

Abbreviations used: AD: Alzheimer's disease; AHA/ASA: American Heart Association/American Stroke Association; AP: anatomic pathology; CERAD: Consortium to Establish a Registry for AD; DSM-V: Diagnostic and statistical manual of mental Disorders version 5; IWG: International Working Group; LBD: Lewy's Body disease; NA: Non-appliable; NINCDS-ADRDA/NINDS-AIREN: National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders

Association / National Institute of Neurological Disorders and Stroke – Association Internationale pour la Recherche et l'Enseignement en Neurosciences; NIA-AA: National Institute on Aging and Alzheimer's Association; PET: positron emission tomography; VASCOG: Vascular Cognitive Disorders Study Group of the International Society for Vascular Behavioural and Cognitive Disorders VCING: Vascular Cognitive Impairment Neuropathology Guidelines; VICCCS: Vienna Institute of Clinical and Cognitive Research on Cerebrovascular and Cognitive Syndromes.

**BMs include: amyloid PET imaging and/or fluid BMs such as amyloid and/or phosphorylated tau fragment ratios (e.g., CSF p-tau181/Aβ42, CSF t-tau/Aβ42, CSF Aβ42/40; plasma pTau217)).*

Several efforts have been made to define AD-CVD clinically, NPS, radiologically and neuropathologically (55). Traditionally, it has been proposed that AD-CVD should be suspected in cases where a patient with CVRF presents atypical AD-like symptoms or when a patient diagnosed with VCI displays clinical features typically associated with AD (51).

It has been claimed that AD-CVD cases have distinguishing features in comparison to pure AD cases, such as more comorbidities and a worse prognosis (56), as suggested by a study in which patients with subcortical vascular dementia and a positive amyloid PET progressed faster than the amyloid negative ones (57). It has also been stated that AD-CVD patients tend to have a higher prevalence of gait disturbances and neuropsychiatric symptoms such as apathy, depression or agitation (58). From a NPS perspective, AD-CVD patients often perform worse in attention, executive function and visuoconstructional abilities and better in memory than those with" pure" AD (59). Some researchers propose that the integration of statistical modelling techniques that combine MRI and NPS test performance may enhance the classification of patients across the neuropathological and clinical AD-CVD spectrum.

However, AD and VCI overlap significantly in both neuropathological and clinical dimensions. Notably, even comprehensive clinical and NPS evaluations may be incapable of definitively distinguishing these conditions with high certainty. Thus, some argue that "pure" cases of either AD or VCI are uncommon, proposing instead that most cases fall along a continuum or spectrum encompassing both pathologies (60).

Biomarkers in AD, VCI and AD-CVD

A BM is a parameter that can be objectively measured in vivo and gives information about a normal or pathological biological process (61).

Although research on BMs for evaluating cognitive impairment has primarily focused on AD, there is growing interest in identifying BMs for other conditions that impair cognition, including VCI. However, outside of AD, well-established recommendations for the clinical application of BMs remain limited.

Neuroimaging

Structural MRI is a critical tool for evaluating CVD and VCI, as well as for assessing cognitive impairment more broadly. It enables accurate evaluation of the degree and pattern of brain atrophy and helps to exclude other lesions that may contribute to the cognitive impairment (e.g. cerebral tumours). Accordingly, MRI is the first-line neuroimaging modality recommended across several guidelines for evaluating AD, VCI and cognitive impairment, including the one by the American College of Radiology (62). Alternatively, a brain computed tomography (CT) scan -though less sensitive, especially for detecting atrophy patterns and svCVD- may be acceptable when MRI is not feasible (e.g., due to the presence of a non-compatible pacemaker, or in emergency settings).

Table 3 provides a summary of the most common sequences used in cerebral MRI.

Table 3. Most frequently used MRI sequences in neuroimaging (68,69)

MRI sequence	Characteristics	Uses in neuroimaging

T1 weighted	The "anatomical" sequence.
	It can be enhanced with contrast (gadolinium).
	Fat suppression techniques can also be applied when necessary (particularly in post-contrast imaging or in fat-rich regions).
	The typical signal intensities are:
	- Fluid (e.g., CSF): low (black).
	- Muscle: intermediate (grey)
	- Fat: high (white).
	- Brain
	  o Grey matter: intermediate (grey)
	  o White matter: hyperintense compared to grey matter (white-ish).
		To calculate brain tissue volumes.
		To distinguish lacunes from PVS.
		Gadolinium-based contrasts can be useful for highlighting areas of BBB or increased vascularity (like in tumours or inflammation).

T2 weighted	The typical signal intensities are:
	- Fluid (e.g., CSF): high (white).
	- Muscle: intermediate (grey)
	- Fat: high (white)
	- Brain
	  o Grey matter: intermediate (grey)
	  o White matter: hypointense compared to grey matter (dark-ish)
	Suppression techniques can also be used:
	- STIR (Short tau Inversion Recovery): supresses fat signal
	- FLAIR (Fluid-Attenuated Inversion Recovery): suppresses the high signal of CSF.
		Well-suited to detect fluid content.
		It is therefore useful to detect oedema, inflammation, infection, tumours, demyelination. In CVD, it is valuable in differentiating lacunes and chronic infarcts from WMH and PVS.
		FLAIR enhances visibility of PV and cortical lesions.

Diffusion weighted	It assesses the facility with which water molecules move within a tissue, making it useful for assessing cellularity (e.g., in tumours), intracellular changes (e.g., ischaemia) and extracellular oedema. Accordingly, the terms "restricted"and "facilitated" diffusion are used to indicate whether water can move less or more easily than expected for that tissue. Diffusion-weighted sequences typically include: - **Diffusion-weighted imaging (DWI):** Areas of increased signal may reflect restricted diffusion, as seen in many acute pathologies such as ischaemic stroke or abscesses (due to high cellularity or cytotoxic oedema). However, increased signal may also result from the **T2 shine-through effect**, where tissues with intrinsically high T2 signal (e.g., CSF, some tumours) appear hyperintense on DWI, despite not having true diffusion restriction. This occurs because DWI sequences retain some T2-weighted signal contribution. - **Apparent Diffusion Coefficient (ADC)** maps: These maps removethe T2 -weighted component of DWI, allowing a more accurate assessment of diffusion. Areas of true restricted diffusion show low ADC values.	To detect acute ischemic lesions (usually up to several weeks after the event).
Susceptibility sensitive	There are several alternatives sensitive to different substances (calcium, blood). Some examples are: - Gradient-Recalled Echo (GRE) T2-weighted* - Susceptibility-Weighted Imaging (SWI): A high-resolution 3D sequence based on GRE principles. It is more sensitive and specific compared to conventional GRE. However, it requires longer acquisition times and is more susceptible to motion artifacts. - Quantitative susceptibility mapping (QSM): provides quantitative assessments of tissue magnetic susceptibility rather than solely qualitative data.	GRE T2*: blood products such as haemorrhages (including ICH, CMBs, SAH or cSS), iron deposition (common in neurodegenerative diseases), calcifications. QSM may help in differentiating calcification from haemorrhage and in better characterizing CMBs and vascular malformations.

Neuroimaging for AD

While certain MRI patterns are suggestive of AD- such as temporally predominant atrophy in the early stages of late-onset AD- these changes are relatively non-specific. Similar patterns of atrophy can also be observed in other conditions, including limbic-predominant age-related TDP-43 encephalopathy (LATE), FTD, and mesial temporal sclerosis (which can be the consequence of different processes).

However, recent advances in neuroimaging have enhanced the specificity and sensitivity of structural evaluations. These include ultra-high-field MRI systems (e.g., 7-Tesla

scanners), which allow for detailed assessment of small brain regions, as well as automated volumetric analysis software capable of identifying and tracking subtle structural changes. Additionally, functional MRI enables the detection of early functional alterations in cases where structural changes may not yet be apparent. Collectively, these innovations facilitate the more accurate identification of disease-specific patterns of brain atrophy in neurodegenerative conditions such as AD and PD (63). As these technologies become more fully integrated into clinical practice, they are likely to enhance the precision of differential diagnosis based solely on MRI findings.

Molecular imaging techniques, particularly those based on nuclear medicine such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), can also support the diagnosis in selected cases. For instance, amyloid PET provides an alternative to other amyloid BMs, while FDG-PET -using fluorodeoxyglucose- serves as a marker of neurodegeneration by detecting regions of hypometabolism, which may be present in early disease stages before significant atrophy occurs. DaTSCAN (dopamine transport scan) -a type of SPECT using Isoflurane I-123 as a tracer- is employed to assess dopamine transporter availability in the brain. It can aid in the differential diagnosis with disorders characterized by dopaminergic degeneration, such as PD and LBD (64).

Neuroimaging for VCI and svCVD

Many different types of cerebrovascular lesions exist and as mentioned, MRI remains the Gold Standard for their evaluation. A summary of the most frequent ones and their radiological features when evaluated through MRI is provided in Table 4. Additionally, some lesions associated with svCVD are illustrated in Figure 1. As stated above, many of them can lead to cognitive impairment, regardless of whether they produce acute symptoms or not.

Table 4. Common cerebrovascular lesions and associations with VCI

Lesion	Subtypes/location	Main radiological features (65,248)	Other features/comments	Significance with regards to cognitive impairment according to VASCOG criteria (31)
Brain infarct	All infarcts	Radiological features vary according to the chronological stage of the infarct. Recent infarcts appear hyperintense on DWI, T2-weighted, and FLAIR sequences, and hypointense on T1-weighted imaging. Over time, the DWI signal typically becomes isointense, reflecting resolution of diffusion restriction. Larger infarcts may undergo cavitation, while smaller infarcts may evolve into lacunar lesions without visible cavitation, or in some cases, may become radiologically undetectable.		
	Lacunar infarct	While the exact definition varies, infarcts up to 15-20 mm in MRI evaluation are usually considered lacunar infarcts.	They are more frequently related to svCVD.	>2 or 1-2 if strategic or associated with extensive WMHs The brainstem and the cerebellum are excluded.
	Large infarct	There is no consensus on the size a "large" brain infarct must have to be considered as such.	Usually due to problems arising in large vessels and/or its branches and/or the heart.	≥ 1 or 2 (depending on size and location).
	Other common specifications according to location	Subcortical or deep		
		Territorial	Often indicates the origin of the problem is in a large vessel or in the heart (cardioembolism).	
		Cerebellar		
		Brainstem		
Presumed vascular origin lacunae		Round or ovoid, subcortical, fluid filled (CSF-like) cavity. Size is usually 3-15mm.	While they can result from lacunar ischaemic or haemorrhagic infarcts, they can be related to several causes, and they can also appear de novo in healthy tissue. They have been associated with an increased risk of future stroke (44).	
WMHs of presumed vascular origin*	Periventricular, subcortical, and brainstem	"Hyperintense on T2weighted (preferably FLAIR) MRI sequences and typically symmetrical between hemispheres" (65).		Extensive and confluent WMHs as defined by grades 2 or 3 in Fazekas scale**.
CMBs	Typically, "lobar"(cortical or juxtacortical structures) vs "deep" (brainstem, deep grey matter, deep white matter). Cerebellum is usually considered aside.	Lesions of low signal on MRI T2 or susceptibility-weighted sequences (65). Usually, diameter is 2-5 mm. Although there are inconsistencies in the literature, usually if the diameter is >5-10 mm is already considered a "macro haemorrhage" (or ICH) (170).	They have been used as a marker of cerebral blood vessels' frailty. Strictly lobar CMBs have good diagnostic accuracy for CAA in older patients (>65 years), as enshrined in the Boston 2.0. criteria (36). Conversely, deep CMBs are more related to arteriolosclerosis, but overlap exists. Generally, no acute clinical symptoms. The burden of lobar CMBs has been associated with the risk of recurrence after a ICH, with >5 CMBs at baseline bearing a recurrence incidence of 51% (follow-up 32.9 +/- 24.0 months) (39).	Not considered.
(spontaneous) ICH		Overall, its most common cause is svCVD, either by arteriolosclerosis or CAA. They are larger than CMBs, often have several centimetres.	In contrast to CMBs, they are often symptomatic and even life-threatening.	≥ 1 or 2 (depending on size and location).
cSS	It refers to "chronic blood products in or overlying the superficial cortex"(65). Classified in focal (involvement of ≤3 sulci) or disseminated (involvement of ≥4 sulci, often across multiple lobes or both hemispheres).	Linear hypo intensity in T2 weighted gradient echo or other blood-sensitive sequences.	It can have several aetiologies, such as trauma or SAH. The latter may be secondary to CAA in older patients (>65 years), so it is recognised as a diagnostic BM of CAA in the Boston 2.0. criteria (36).	Not included.
(enlarged)*** PVS	PVS are "extensions of the extracerebral fluid space around arteries, arterioles, veins, and venules as they course from the brain surface into and through the brain parenchyma" (65).	"Fluid filled, round, ovoid, or linear spaces that follow the typical course of small perforating vessels as they go through white or deep grey matter" (65). Similar signal to CSF. Usually, diameter <2 mm.	PVS have recently emerged as a proxy of glymphatic dysfunction (215). They increase with age and CVRF. When located in the CSO, can also be a sign of CAA, as recognised in the Boston 2.0. criteria (36).	Not considered. However, some studies have associated them with worse cognitive function (249).

Abbreviations: CAA: cerebral amyloid angiopathy; CMBs: cerebral microbleeds; CSF: cerebrospinal fluid; CSO: centrum semiovale; cSS: cortical superficial siderosis; CVRF: cardiovascular risk factors; DWI:

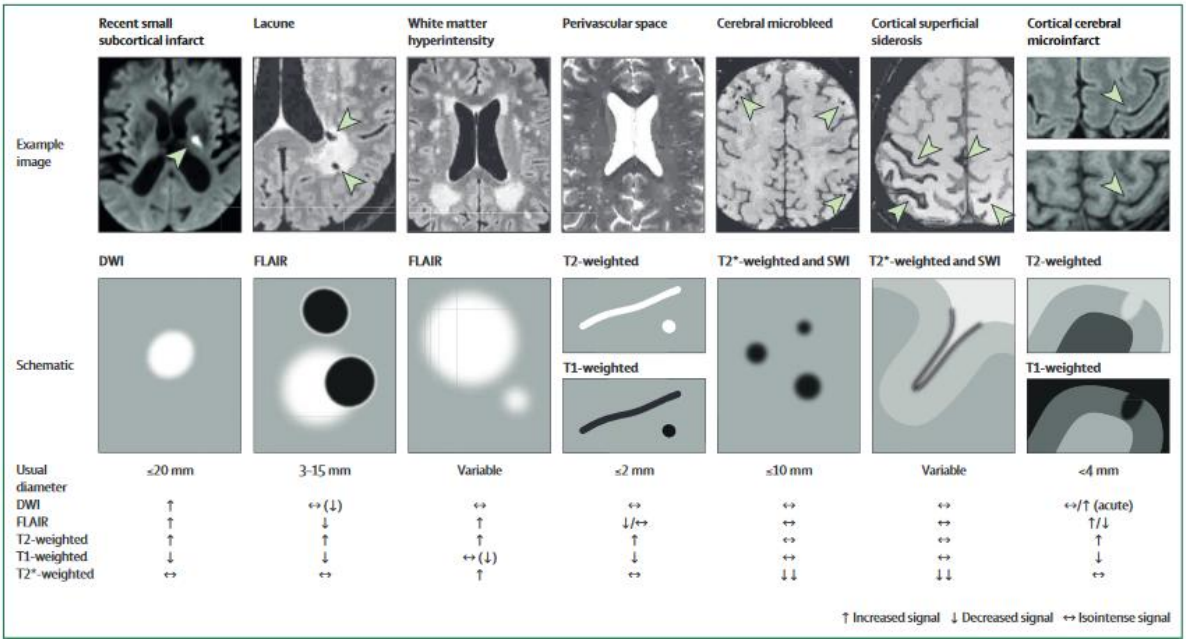
diffusion weighted imaging; ICH: intracranial haemorrhage, MRI: magnetic resonance imaging; PVS: perivascular spaces; SAH: subarachnoid haemorrhage; svCVD: small vessel cerebrovascular disease; VCI: vascular cognitive impairment; WMHs: white matter hyperintensities

**To align with the terminology used in most of the available literature and in the clinical practice, the term "WMHs" will be used to refer to subcortical hyperintensities in any of these three locations. However, STRIVE-2 uses the term "subcortical hyperintensities" rather than WMHs whenever subcortical or brainstem lesions are included (65).*

*** Fazekas score is a visual rating scale that is commonly used for evaluating WMHs. It ranges from 0 to 3, with a score of 0 indicating no or few white matter changes, a score of 1 indicating multiple punctate changes, a score of 2 indicating changes that start to become confluent and a score of 3 indicating changes that are fully confluent (66). However, there are currently no clear recommendations or guidelines for accurately estimating the positive predictive value of WMHs in causing cognitive impairment on an individual basis. Moreover, the prevalence of WMHs increases with age - age- and sex-stratified data have been proposed- and varies significantly between populations for reasons that are still not fully understood (67).*

****The term "enlarged" is not used in the STRIVE-2 criteria (72).*

Figure 1. Main imaging features of some svCVD lesions (extracted from Duering et al. (65))



Another significant challenge is to evaluate svCVD, as it encompasses a broad spectrum of lesion types that may evolve over time, as already mentioned in the VCI sections. svCVD is a dynamic phenomenon which can be observed through MRI, progressing (i.e. one type of lesion may evolve into another type of lesion), regressing or even disappearing (68), independently of stroke history or cognitive symptoms, as shown in Figure 2. However, the intricacies of svCVD evolution (e.g., which lesions will evolve to

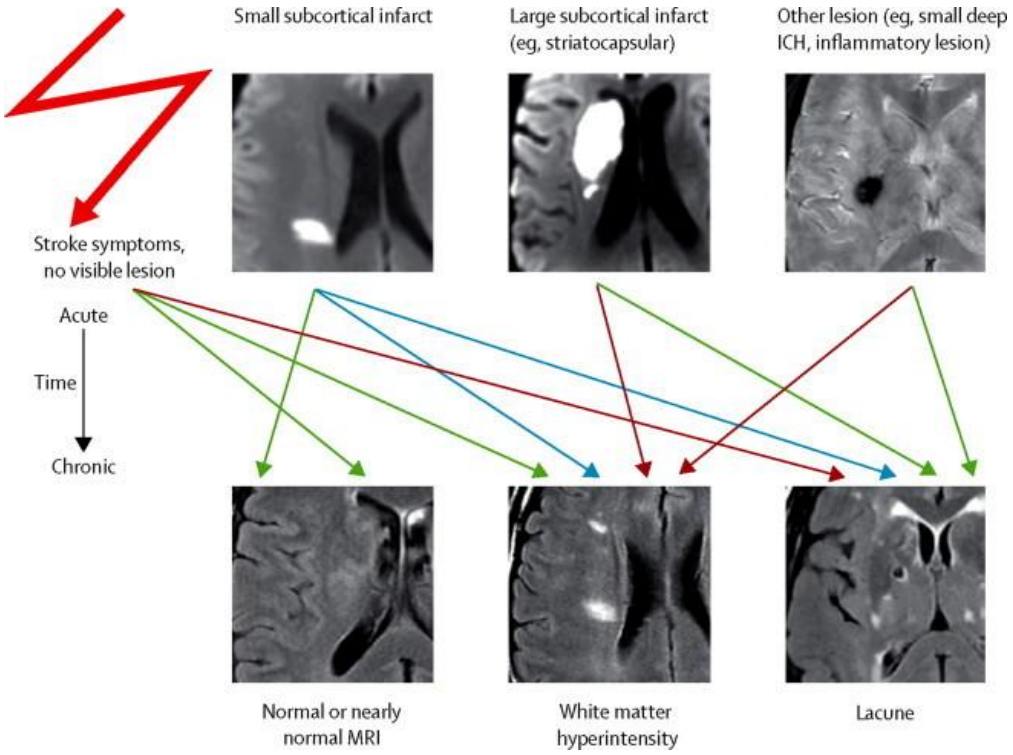
others, and which will resolve) remains elusive. In this regard, the studies by Jiang et al. (68) and Ammirati et al (69) are interesting.

Jiang et al. (68) followed up the WMH burden in 225 individuals immediately after having a minor stroke and after two years of follow up. They found that WMH volume increased in 50.2%, decreased in 32.8% patients and remained stable in 25.8%. They associated progression with having a history of hypertension and a higher svCVD burden at baseline.

Ammirati et al (69) included 51 participants (mean age of 69 ± 8 years) with a carotid stenosis of <70% and no prior history of stroke or TIA and investigated the development of WMHs or CBI over a median follow up of 595 days (IQR: 553-641). In that sample, 78% of which were hypertensive, 73% had dyslipidaemia (higher than in our cohort), and 22% were diabetic (similar to our cohort). Among those who developed at least one new significant WMH lesion, the average number of new lesions was five. Although the Rotterdam Progression Scale could not be directly applied due to the absence of lesion location data, it is likely that a substantial proportion would have met our threshold for 'marked' progression (defined as a Rotterdam score >2.5, as detailed in the Methods section).

In both studies, baseline burden of WMHs was the most important predictor of progression. This aligns with the baseline burden of svCVD as evaluated through neuroimaging being the most important prognostic predictor of VCI after a stroke, as outlined in the VCI subsection.

Figure 2. Potential evolutions of svCVD lesions (extracted from Wardlaw et al.(65))



The Standards for Reporting Vascular Changes on Neuroimaging 1 (STRIVE-1) were published in 2013 with the primary goals of describing the radiological features of svCVD and promoting a standardized vocabulary. Advances in the understanding of svCVD have since then led to the development of STRIVE-2, which updated terminology and included recommendations for their application (65).

Additionally, STRIVE-2 offers a comprehensive summary of svCVD radiological markers, incorporating various visual rating scales, such as Fazekas and Schmidt scale, the Scheltens scale, and Manolio scale, for evaluating WMHs, atrophy, and other radiological signs of CVD.

Furthermore, recognizing the limitations of qualitative assessments, STRIVE-2 also includes automated quantitative imaging markers and techniques for structural and functional brain evaluation. These include diffusion imaging, tissue susceptibility mapping, BBB imaging, and perfusion and flow imaging. The extent of automation status varies depending on the type of lesion and outcome being assessed; fully automated

approaches are well-established and have been clinically validated for certain outcomes, such as brain atrophy and WMHs, while automated methods for others, like cortical CMBs, are still lacking.

Notably, CVD can lead to various patterns of brain atrophy, either because of cerebral infarcts or independently, through changes associated with svCVD. The latter is most commonly manifested as diffuse cortical grey matter loss and has been strongly associated with WMHs burden (70).

AD BMs

BMs are extremely useful to increase diagnostic accuracy, explore potential therapeutic targets and monitor responses to interventions. However, they have been so far relatively poorly studied in co-pathology cases, possibly due to the inherent complexity of mixed cases.

BMs have facilitated a shift in the understanding of AD, which is now understood as a biological entity identifiable through the use of BMs. This biological approach is well reflected in the NIA-AA 2024 criteria (54) (Figure 3 and Figure 4).

Figure 3. Categorisation of biofluid and imaging (extracted from Jack et al. (47)

Biomarker category	CSF or plasma analytes	Imaging
Core Biomarkers		
Core 1		
A (Aβ proteinopathy)	Aβ 42	Amyloid PET
T_1: (phosphorylated and secreted AD tau)	p-tau217, p-tau181, p-tau231	
Core 2		
T_2 (AD tau proteinopathy)	MTBR-tau243, other phosphorylated tau forms (e.g., p-tau205), non-phosphorylated mid-region tau fragments[a]	Tau PET
Biomarkers of non-specific processes involved in AD pathophysiology		
N (injury, dysfunction, or degeneration of neuropil)	NfL	Anatomic MRI, FDG PET
I (inflammation) Astrocytic activation	GFAP	
Biomarkers of non-AD copathology		
V vascular brain injury		Infarction on MRI or CT, WMH
S α-synuclein	αSyn-SAA[a]	

Figure 4. Intended uses for AD BMs (extracted from Jack et al. (47))

Intended use	CSF	Plasma	Imaging
Diagnosis			
A: (Aβ proteinopathy)	–	–	Amyloid PET
T_1: (phosphorylated and secreted AD tau)	–	p-tau217	–
Hybrid ratios	p-tau181/Aβ42, t-tau/Aβ42, Aβ42/40	%p-tau217	–
Staging, prognosis, as an indicator of biological treatment effect			
A: (Aβ proteinopathy)	–	–	Amyloid PET
T_1: (phosphorylated and secreted AD tau)	–	p-tau217	–
Hybrid ratios	p-tau181/Aβ42, t-tau/Aβ42, Aβ42/40	%p-tau217	–
T_2: (AD tau proteinopathy)	MTBR-tau243, other p-tau forms (e.g., p-tau205), non-phosphorylated mid-region tau fragments	MTBR-tau243, other p-tau forms (e.g., p-tau205)	Tau PET
N (injury, dysfunction, or degeneration of neuropil)	NfL	NfL	Anatomic MRI, FDG PET
I (inflammation) Astrocytic activation	GFAP	GFAP	–
Identification of copathology			
N (injury, dysfunction, or degeneration of neuropil)	NfL	NfL	Anatomic MRI, FDG PET
V vascular brain injury	–	–	Infarction on MRI or CT, WMH
S α-synuclein	αSyn-SAA	–	

One significant advancement is the development of blood BMs, facilitated by technologies such as the Single Molecule Array (Simoa) technique**.** Blood BMs are more accessible and cost-effective, and some, such as pTau 217, have demonstrated equivalent or even superior performance compared to well-established CSF counterparts in real-world clinical settings (71,72). For instance, the ratio of blood pTau217 to non-phosphorylated pTau, measured using mass spectrometry, showed to perform as well as or better than CSF Aβ42/40 and pTau181/Aβ42 (71). In contrast, the Aβ42/40 ratio in blood-based assays is less accurate than in CSF when assessed using immunoassays(73). Mass spectrometry assays provide significantly improved measurements of blood-based Aβ42 and Aβ40; however, they are costly and not feasible in routine laboratories.

In the following section the main BMs included in the NIA-AA 2024 criteria will be first reviewed. Next, the most recent IWG-3 criteria will be briefly introduced and contrasted

with the NIA-AA framework. Finally, potential BMs for VCI other than neuroimaging, as well as the current state of AD-CVD biofluid BMs, will be discussed.

The NIA-AA 2024 criteria (54)

These criteria published in the 2024 have classified BMs used in AD into **core AD BMs, BMs of non-specific processes involved in AD pathophysiology, and BMs of AD co-pathologies.** The core AD BMs are further divided into two groups -core 1 and core 2- based on their intended use. The roles of amyloid and tau BMs have been redefined. The presence of core 1 BMs is considered enough to establish the presence of AD. Tau BMs are split into two categories depending on the moment of the disease course on which they appear, designated as T1- included among the core 1 BMs- and T2- included among the core 2 BMs (54).

Core 1 BMs: amyloid pathology

They are surrogates of a process of brain amyloidosis. They are used to establish the diagnosis of AD in the 2024 NIA-AA criteria. They include amyloid PET and biofluid BMs associated with amyloid pathology, which are: CSF Aβ42, and, more specifically, hybrid ratios (pTau/Aβ42 and tTau/Aβ42Aβ42/40), and blood phosphorylated mid-region tau fragments (ptau217, ptau181, ptau231).

Core 1 biofluid BMs become abnormal earlier in the AD continuum than amyloid and tau PET, so they are considered the most sensitive and suitable BMs currently available for detecting pre-symptomatic and prodromal cases.

Hybrid ratios are employed to account for variability between high and low amyloid producers (74). While the pTau/Aβ42 and tTau/Aβ42 ratios are generally considered to perform similarly to the Aβ42/40 ratio, studies by Amft et al. suggest that Aβ42/40

demonstrates superior performance, particularly in terms of specificity. Specifically, the point estimate for Aβ42/40 is 0.82, compared to 0.69 for both alternative ratios (75).

Phosphorylated mid-region tau fragments (p-tau 181, p-tau 217, and p-tau 231), comprised in the T1 category, have shown to correlate better with fluid and PET amyloid BMs than with PET tau. Thus, they are used as a proxy of amyloid plaques in the brain rather than of tau aggregates. In fact, it has been argued that the phosphorylation of these fragments may be a reaction to amyloid plaques. However, it is ultimately a tau marker, as showed by the fact that its levels can raise in amyloid negative individuals. Specifically, they have been seen to increase in other neurodegenerative tauopathies such as Niemann-Pick disease, in which plasma p-Tau217 has been shown to raise similarly to amyloid-positive and tau-negative individuals within the AD continuum(76). Although the increase in pTau levels is more subtle in the Niemman-Pick and the amyloid positive and tau negative groups than in the amyloid and tau positive AD individuals, this raises concerns regarding the specificity of pTau as a BM for AD.

There are several isoforms of phosphorylated tau (pTau), and various studies have proposed that differences exist in their performance as BMs (73). Specifically, blood pTau217 has shown to have an excellent accuracy in detecting Aβ (area under the curve (AUC) 0.92-0.96; 95% CI, 0.89-0.99) and tau (AUC, 0.93-0.97; 95% CI, 0.84-0.99) pathology in three observational cohorts formed by individuals, with and without cognitive impairment, who had a positive amyloid status by PET or CSF (72). It has also demonstrated to be very accurate in distinguishing AD from other neurodegenerative disorders (77). In this sense, it has shown to outperform all the other studied blood BMs- pTau181, pTau 231, Aβ 42/40, GFAP and NfL - and their optimal combinations. Furthermore, as previously noted, pTau217 levels demonstrated a consistent increase over time across individuals diagnosed with AD, which was more pronounced in individuals with a positive tau status compared to those who were amyloid-positive but tau-negative. Higher levels of pTau217 predict the conversion from CU to MCI or from

MCI to AD dementia in patients with a positive cerebral amyloid PET, thus having certain prognostic potential (72). In contrast, the levels of Aβ42 and Aβ40 remain relatively stable throughout the disease course, limiting their utility for monitoring disease progression (73).

Core 2 BMs: tau pathology

Core 2 BMs mainly relate to tau pathology. They include PET-tau and T2 tau biofluid BMs, which includes microtubule-binding region residue 243 (MTBR-tau243) and other non-phosphorylated tau fragments.

In contrast to core 1 BMs, they are not widely implemented in the clinical practice yet.

They provide prognostic information and allow biologically staging each individual within the AD continuum.

They correlate more strongly with clinical symptoms than core 1 BMs, and have shown to exhibit a negative correlation with cognitive function as assessed by the MMSE (78).

MTBR-tau243 reflects the presence of insoluble tau aggregates in the medial temporal regions, which precedes tau deposition in the cortex. Tau deposition in the cortex is better defined by PET-tau and indicates a more advanced stage of the disease (54).

In blood samples, an ultrasensitive assay utilizing the TauJ.5H2 antibody for detecting a specific exon sequence of brain-derived total tau has demonstrated the ability to accurately discriminate between individuals with AD and those without, as confirmed by CSF BMs for AD and postmortem autopsy findings. Moreover, it has shown specificity to AD type neurodegeneration, and its performance is not affected by other concomitant pathologies. As a result, it has the potential to facilitate the determination of AD-specific tau pathology in a blood sample. Thus, if combined with phosphorylated tau fragments and non-specific blood BMs of neurodegeneration, such as NfL, it would allow to establish the ATN using exclusively blood-based BMs in the near future (79).

BMs of non-specific processes involved in AD pathophysiology

This category encompasses the structural MRI and nuclear imaging techniques already commented above, as well as biofluid BMs such as glial fibrillary acidic protein (GFAP) and neurofilament light chain (NfL**)**. Notably, beyond their diagnostic utility, many of these modalities also offer substantial value for prognostic assessment.

In contrast to phosphorylated fragments of tau, the main source of blood total tau seems to be predominantly peripheral (non-brain) rather than the CNS except in cases where there is a high t-tau release at the CNS, such as in certain AD cases or other neurodegenerative pathologies, TBI or acute stroke (80). more associated with non-specific neurodegeneration than with tau AD pathology. Furthermore, in contrast to the Aβ42/40 ratio and pTau isoforms, there is a poor correlation between CSF total tau and blood total tau. Therefore, CSF and blood t-tau should be interpreted as a non-specific BM of neuronal injury, and possible peripheral sources of t-tau should also be considered depending on the clinical context.

GFAP and NfL are non-specific BMs reflecting glial and neuronal injury, respectively. Furthermore, it has been hypothesized that they may play active roles in neurodegeneration and neuroinflammation, positioning them as potential therapeutic targets. In the 2024 NIA-AA AD diagnostic criteria(54), NfL is considered a BM of degeneration of the neuropil (N) and GFAP is considered a BM of neuroinflammation (I) and astrocytic activation.

GFAP is overexpressed, along with other proteins, by astrocytes in pathological conditions, including amyloid deposition, which may explain its strong correlation with cerebral amyloid pathology. GFAP has demonstrated to consistently distinguish between AD and controls, as well as between amyloid positive and negative individuals with MCI (81). It may be also used as a marker of astrocyte dysfunction and may correlate with

glymphatic system dysfunction. Bellaver et al. associated higher astrocyte reactivity, as assessed by GFAP levels, with higher future tau burden, as assessed by PET (82).

Importantly, though, neither NfL nor GFAP is specific to AD, as they can be elevated in other neurodegenerative dementias (73) and in non-degenerative conditions such as VCI, HIV-associated dementia and multiple sclerosis (MS). In VCI, elevated plasma and CSF levels of GFAP and NfL have been associated with a higher WMH load and an increased vascular lesions burden, including cerebral infarcts and CMBs(83).

Interestingly, GFAP has shown to be a highly specific brain protein and performs better in blood than in CSF (73). When interpreting GFAP levels, it is crucial to account for demographic factors, particularly age and sex. Probably because, as mentioned earlier, there is a physiological astrogliosis with increasing age, GFAP levels tend to be higher in older individuals. Women generally exhibit elevated levels. ApoE carriership status should be also considered, regardless of clinical disease status, as individuals with ApoE4 genotype have higher levels of NfL, GFAP, and tTau (84).

Despite their non-specificity, GFAP and NfL are sensitive BMs throughout the disease continuum, including early stages of neurodegeneration. GFAP has been shown to precede more specific AD BMs, such as amyloid and tau, in several studies (81),while NfL levels begin to rise during the preclinical stages of the disease (73). Consequently, these BMs could serve as useful screening tools to differentiate neurodegenerative diseases, such as the behavioural variant of FTD, from conditions that do not influence these BMs, such as primary psychiatric disorders.

Moreover, like pTau in AD cases, GFAP and NfL levels increase throughout the course of various neurodegenerative conditions and have been correlated with poorer performance in NPS assessments, a higher rate of conversion from MCI to dementia, and greater atrophy on structural neuroimaging (81,85). Thus, they may be valuable for tracking disease progression and monitoring responses in RCTs of potential DMTs.

However, the literature presents conflicting results; for instance, Mattson et al. found that, unlike pTau217, increases in blood NfL levels were not significantly different between cognitively unimpaired individuals and those with MCI, regardless of their amyloid status (86).

The third version of the International Working group criteria (IWG-3) (87)

In contrast to the 2024 NIA-AA criteria, the IWG-3 criteria emphasize the importance of incorporating the clinical phenotype and factors related to individual resilience (e.g. comorbidities and neurologic co-pathology, educational level, genetics) into the categorization of individuals. That is, they require a clinical phenotype consistent with AD or the presence of additional factors -such as highly penetrant autosomal dominant genetic mutations associated with a near 100% lifetime risk of clinical AD- for an AD diagnosis to be made. In this line, asymptomatic individuals with positive AD BMs without any additional factors that confer further risk (e.g., Down syndrome or homozygosity for the APOEe4 allele). may be categorised as “at risk” of AD, being the lifetime risk and timing of progression dependent on these modulating factors. This is illustrated by the fact that in the follow up of a cohort of cognitively unimpaired individuals with positive amyloid BMs and a mean age of 77 years at baseline, only 17% developed symptoms consistent with AD after 5 years of follow-up (88). Further research is needed to better understand and quantify the impact of such factors on disease trajectory.

VCI biofluid BMs

As mentioned in the corresponding section, neuroimaging and, specifically, cerebral MRI, remains the Gold Standard for evaluating CVD and/or VCI in vivo. However, some

efforts have been made to develop biofluid BMs for VCI in order to complement the MRI evaluation and overcome some of its limitations.

Research on biofluid BMs for VCI has predominantly focused on the various pathological processes involved in this condition. These include endothelial dysfunction (e.g., placental growth factor [PlGF]), blood-brain barrier (BBB) breakdown (e.g., soluble platelet-derived growth factor receptor β [sPDGFRβ], matrix metalloproteinases [MMPs]), the clotting pathway (e.g., fibrinogen, von Willebrand factor, thrombomodulin, plasminogen activator inhibitor-1 [PAI-1]), and inflammation (e.g., interleukins IL-6, IL-1β, IL-18, C-reactive protein, tumor necrosis factor [TNF], insulin-like growth factor 1 [IGF-1], or receptor for advanced glycation end products [RAGE]) (83). Some BMs have been specifically associated with svCVD and may offer the potential to further subcategorize VCI (83).

Although endothelial dysfunction and BBB breakdown occur in CVD and in AD, several studies have reported statistically significant differences in the levels of BMs that assess these processes between the two conditions. This discrepancy may indicate that in "pure" AD these pathological changes occur to a lesser extent than in CVD or mixed cases. For instance, variations in the CSF-to-serum albumin ratio have been documented across different causes of dementia, including AD, VaD and AD-CVD. Notably, the ratio values in the latter two groups were found to be higher than those in the AD group (89). As a mode of example, the MMP index- defined as the ratio of MMP levels in CSF to MMP levels in plasma- has demonstrated the ability to distinguish VCI from AD. Moreover, the combined assessment of the MMP-2 index with MMP-3 activity can effectively differentiate svCVD from other forms of VCI with high specificity(90,91).

Despite conflicting results regarding the use of neuroinflammation BMs, such as IL-6, in the context of VCI (83), some promising BMs have been found, such as sTREM2.

Soluble triggering receptor expressed on myeloid cells 2 (sTREM2) has emerged as an interesting fluid BM. TREM2 is a protein that plays a crucial role in the inflammatory responses of microglia, specifically activating them in a unique manner. However, it is important to note that microglial activation is a complex process involving multiple pathways.

In AD, the levels of sTREM2 may vary throughout the course of the disease: in CSF, sTREM2 levels increase during the preclinical stages, peak just prior to the onset of the prodromal phase, and subsequently decline during the dementia phase (92). This variability across the AD continuum may contribute to the conflicting results regarding its performance in AD, rendering its utility as a diagnostic BM for the disease controversial. While most studies have focused on sTREM2 in CSF, blood sTREM2 has been associated with blood AD BMs - such as p-tau-181, t-tau and Aβ -42 (93)- and with positive PET-tau, but also with WMHs (94) and cardiovascular outcomes such as ischaemic heart disease (IHD) and higher mortality and risk of cardiovascular events after a stroke (95,96). Conversely, lower sTREM2 levels have been linked to VaD in a Chinese population (97).

Moreover, variants in the TREM2 gene have been implicated in several neurodegenerative diseases. Specifically, heterozygous variants have been identified as genetic risk factors for both AD and FTD, while homozygous variants can lead to rare and severe early-onset neurodegenerative dementias, such as Nasu-Hakola disease (98). However, further exploration is needed to elucidate the associations between TREM2 variants and the risk of non-AD neurodegenerative diseases.

Like GFAP, sTREM2 levels are influenced by demographic factors such as sex, with women generally exhibiting lower concentrations.

BMs of oxidative stress like lipoprotein-associated phospholipase A2 (Lp-PLA2) reflect atherosclerosis rather than VCI. However, high serum levels of Lp-PLA2 have been

associated with severe WMHs and covert brain infarcts (CBIs) (99). Higher levels of homocysteine -a metabolite involved in oxidative stress pathways- have been associated with VCI and various vascular lesions, including WMHs, CBI and CMBs.

Matrix Gla Protein (MGP) is a vitamin K-dependent protein that inhibits vascular calcification through several mechanisms, and it is overexpressed in atherosclerotic vessel walls(100). To be biologically active, it must undergo carboxylation and phosphorylation, which depend on vitamin K. Uncarboxylated MGP (ucMGP), an inactive form of MGP, has been proposed as a BM of vascular calcification. Lower serums of ucMGP have been, though inconsistently, associated with cardiovascular mortality in patients with high cardiovascular risk- specifically, with history of myocardial infarction, stroke or coronary artery disease or with chronic kidney disease (100). A case-cohort study with 1406 subjects did not find an association between ucMGP levels and coronary heart disease or stroke (101). Lower levels of another inactive form, dephosphorylated-ucMGP, have been associated with worse cognitive performance in patients with chronic kidney disease (102).

AD-CVD biofluid BMs

Studies specifically assessing BMs in the AD-CVD group are scarce. One of the main challenges in this respect is that, given that AD and VCI share numerous pathological pathways, many BMs cannot reliably differentiate between these two conditions. For instance, Eckerström et al. only found slightly lower levels of Aβ42, pTau, and tTau in the AD-CVD group compared to the AD group (103). Similarly, and despite some differences have been observed, BMs assessing amyloid deposition (core 1 AD BMs, including biofluid BMs and PET amyloid) cannot reliably distinguish between AD and CAA (21).

Thus, AD-CVD presents a significant challenge in the field of BMs that warrants further exploration. Ideally, future research should focus on identifying BMs that can specifically target the distinct pathological pathways of each condition, potentially allowing for a more accurate assessment of their individual contributions to a patient's clinical presentation. Alternatively, an appropriate combination of BMs from different modalities (e.g., imaging and biofluids) may prove useful in achieving this goal.

Other potential biofluid BMs for AD and/or VCI

Many other potential BMs are being investigated, such as TAM receptors (Tyro3, Axl, and MerTK; a family of receptor tyrosine kinases), monoamines, and blood circulating microRNAs (104–106). Although retinal BMs, such as retinal thickness and volume and vascular retinal BMs, are still under investigation and data regarding their sensitivity and specificity is lacking, they are promising, since they have the potential of simultaneously assessing both CVD and neurodegenerative diseases (107).

CVD and AD association: potential mechanisms

The prevalence of the association between CVD and neurodegenerative diseases is higher than would be expected by chance (108). Several potential explanations have been proposed.

First, certain conditions, such as CAA and AD, may exhibit both neurodegenerative and cerebrovascular components. Further, there is an increasing body of literature about the early cerebrovascular dysfunction in the AD pathophysiology (109,110). Clinical and preclinical studies suggest that cerebrovascular dysfunction, including changes in the

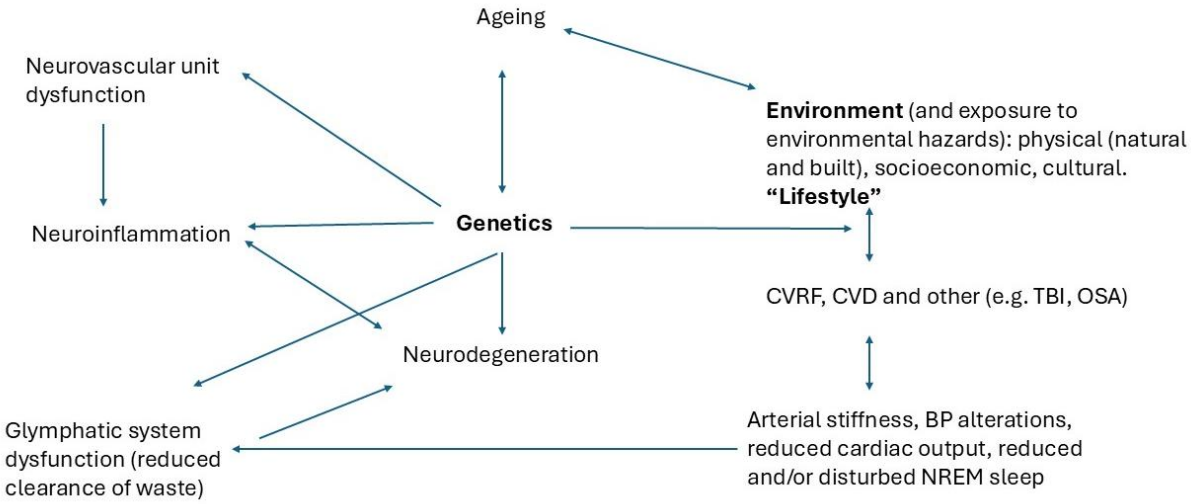
neurovascular unit that will be further explained below, may precede the development of AD neuropathology (110).

Second, the presence of one pathology may influence the development, expression and outcomes of the other. For instance, in a cohort of cognitively unimpaired or MCI individuals with a mean follow-up of 4.68 years, a higher CVD burden was associated with an increased risk of AD, as evidenced by MRI-assessed hippocampal atrophy, lower amyloid levels in CSF, and reduced cognitive scores (57).

In this context, some researchers propose that the AD-CVD association arises because the presence of CVD lowers the clinical threshold for detecting AD, and vice versa—a perspective referred to as the "additive" hypothesis. Alternatively, others argue that shared genetic and environmental risk factors, as well as common pathophysiological processes that interact synergistically, are the primary contributors to the frequent co-occurrence of CVD and AD, a concept known as the “interactive” hypothesis (111).

Aligned with the interactive hypothesis, the following section will summarize key pathophysiological pathways and environmental and genetic risk factors that, along with ageing, are considered to drive the association between CVD and AD. The great number of factors involved -as summarised in Figure 5- could be an explanation for the great phenotypical heterogeneity among conditions impairing cognition, including AD, VCI and AD-CVD.

Figure 5. Pathways involved in neurodegenerative processes and CVD and their interaction



The neurovascular unit

The neurovascular unit is formed by endothelial cells, glial cells, neurons and the extracellular matrix (83). It has a fundamental role in maintaining brain health through the clearance of toxins, immune trafficking, and the maintenance of blood-brain barrier (BBB) integrity and proper permeability. The development of new imaging techniques and other assessment methods, like dynamic contrast-enhanced MRI imaging (112), has helped to better understand and define its several roles.

The dysfunction of the neurovascular unit has been termed "microvascular disease" and may underlie many of the svCVD cases. If the well-functioning of the BBB is impaired, there is an increased cross-over of neurotoxins and inflammatory factors. These, through enhancing oxidative stress and neuroinflammation, reinforce the BBB breakdown and cause damage to neurons and glia, resulting in myelin breakdown and white matter damage, which may be seen as WMHs in MRI (113). The proper functioning of white matter connectivity is increasingly recognised as a mainstay of cognition and critical for maintaining a good adaptability to changing demands (114). WMHs are associated with

an increased risk of future stroke, stroke severity, cognitive decline, death, dependency, impaired gait, and neuropsychiatric symptoms (115,116).

WMHs are thus a radiological sign of neurovascular dysfunction and have important clinical implications, although they can also reflect distinct -though often associated- processes such as neuroinflammation (114).

Beyond being a sign of CVD caused by traditional CVRF such as smoking, diabetes and hypertension, WMHs, and neurovascular unit impairment more broadly, can be caused by a wide range of both acute and chronic conditions such as stroke, traumatic brain injury (TBI) and inflammatory and neurodegenerative diseases, including multiple sclerosis (MS), AD (117), Parkinson disease (PD) and Amyotrophic Lateral Sclerosis (ALS) (112), and environmental hazards, like AP (118). In the same line, several genes associated with neurodegenerative diseases, including PD, Huntington's disease, ALS and AD, have been shown to impair BBB (117). In neurodegenerative conditions, WMHs has been seen to occur in the hippocampus and other cerebral regions in early AD, before brain atrophy and dementia occur (119).

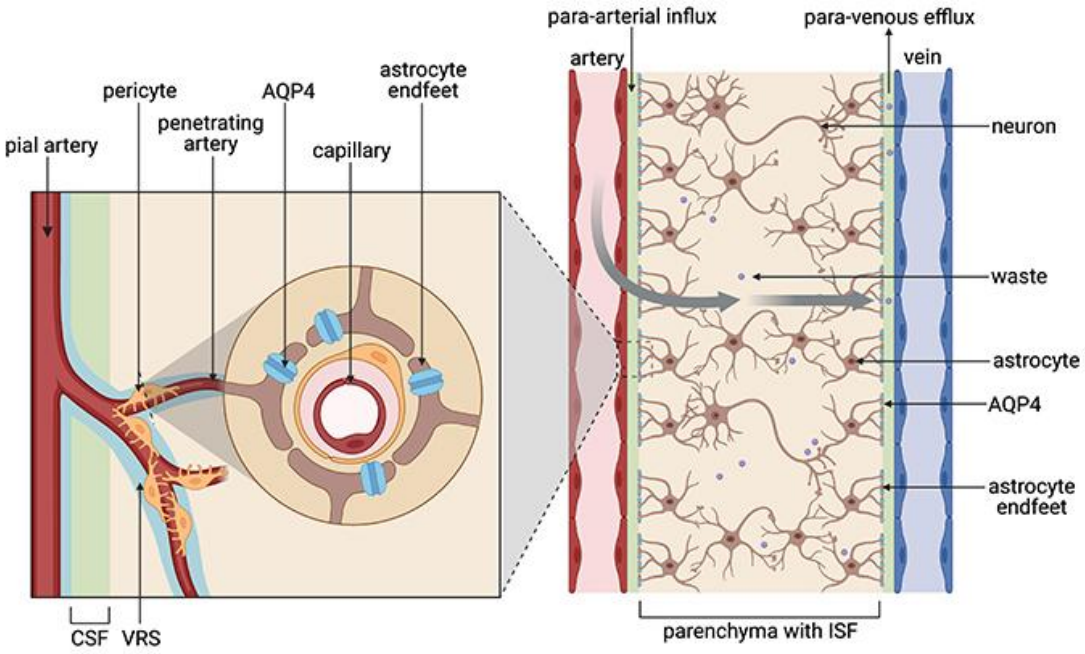
This is illustrated by postmortem studies of patients with AD and a significant WMH load that showed a striking absence of other cerebrovascular lesions (109). Down Syndrome AD (DSAD) cohorts – being Trisomy 21 one of the strongest genetic risk factors for AD (38)- also support this association, since they often exhibit a substantial WMH load despite tending to have a low prevalence of traditional CVRF (except for obesity and sleep apnoea disease) (120). In DSAD and AD cases more broadly, WMHs may be a sign of an underlying CAA, in which, likely, several mechanisms lead to WMHs, including disruptions in the well-functioning of the neurovascular unit and neuroinflammation (38,121).

The glymphatic system

Similarly to WMHs, PVS have long been regarded as secondary to svCVD (whether associated with CVRF or CAA). However, PVS or “enlarged perivascular spaces”, which indicate architectural disruptions in the perivascular space, are now recognised as well as a proxy of the glymphatic system dysfunction.

The glymphatic system is as an astrocyte-regulated mechanism of brain-fluid transport, whose main known function is the removal of waste products of the brain through the perivascular spaces- formed by the vascular end feet of astrocytes. More specifically, CSF is propelled from the subarachnoid space throughout the penetrating arteries into the perivascular spaces by CSF pressure gradients, generated by arterial pulsation and respiration. Slow-wave activity also contributes to the CSF movement along the glymphatic system, which relates to the fact that the glymphatic system is active, or “turned on”, during the NREM deep phases of sleep (122). From perivascular spaces, it enters- and travels through- the neuropil and interstitium. The influx of CSF from the perivascular spaces into the interstitium is regulated by aquaporin (AQ) 4, water channels located in the astrocytes end feet. From there it is propelled out to the cervical lymphatic system, with which the glymphatic system is connected. A schematic representation is provided in Figure 6.

Figure 6. The glymphatic system (extracted from Hazzard et al. (123))



The relatively recent description and study of the glymphatic system have been facilitated by the development of techniques that allow for its in vivo assessment in both animal and human models. Diffusion Tensor Image Analysis ALong the Perivascular Space (DTI-ALPS) MRI sequence, primarily focused on PVS assessment, offers a non-invasive way for evaluating glymphatic system. As with WMH, PVS are not specific to glymphatic dysfunction and may also result from svCVD. However, different regional patterns may help to suggest the most likely underlying pathology. For example, severe PVS located in CSO are more specific to CAA than PVS located in the basal ganglia (40).

Additionally, other MRI and fluid parameters may serve as useful BMs of glymphatic system dysfunction. devices using repeated electrical impedance spectroscopy have been shown to be a promising technique to track the glymphatic system function in humans, and both AQP4 CSF levels and blood GFAP levels have been linked to the dysfunction of the glymphatic system (124) .

The impaired clearance of certain proteins- including misfolded and aggregated proteins such as phosphorylated tau, amyloid-beta and alfa-synuclein- through the glymphatic

system leads to their accumulation in the brain and in the brain vessels. While the physiological process of astrogliosis with increasing age might lead to certain amyloid accumulation that may be considered normal (e.g. up to 30 centiloids is considered normal in amyloid PET scans of the elderly), a significant accumulation of these proteins along with a stagnant glymphatic flow promote their aggregation, which is a central hallmark n many neurodegenerative diseases, including AD (125). Accordingly, DTI-ALPS parameters and AQP4 CSF concentrations have been found to be significantly different between AD, non-AD dementias, stable MCI and cognitive healthy individuals, and correlated with cognitive outcomes and structural changes of MRI damage (126,127). In the same line, glymphatic dysfunction has thus been proposed as a common pathway to neurodegenerative diseases (125) and has been associated with an increased risk of processes such as AD (128), PD or progressive cognitive impairment after TBI (129).

Various factors and conditions might impede the proper functioning of the glymphatic system.

Cardiovascular conditions that lead to arterial wall stiffness—such as chronic hypertension -or to reduced cardiac output -such as myocardial infarction sequelae or arrhythmias- can diminish arterial pulsatility and thereby impair glymphatic flow. Consequently, they may be key contributors to the observed association between neurodegenerative diseases and cardiovascular conditions, including AD and CVD.

Conditions or processes that shorten or disturb the NREM N3 sleep phase can affect the glymphatic system’s function, such as ageing or obstructive sleep apnoea, which is also a recognised CVRF (130).

Genetics is likely to play a role too; certain AQ4 polymorphisms have been linked with the glymphatic system dysfunction in animal and human models (131).

Neuroinflammation

Neuroinflammation is a highly complex process principally mediated by the microglia, specialized immune cells of the central nervous system (CNS). In addition to AD, it has been involved in the development of many neurological diseases, including PD, ALS and MS (132).

Existing genetic, animal and BM data suggest that neuroinflammation generally exerts a beneficial effect in the early stages of disease. However, when neuroinflammation is sustained over time, it may lead to harmful consequences by damaging BBB, impairing CSF flow, altering astrocytes and mislocating AQ4 receptors (132). In fact, it has been stated that chronic neuroinflammation caused by the presence of AD related neuropathology is the true driver of AD progression rather than the presence of the amyloid plaques and tau tangles by themselves(133). The shift from the M2 (mainly anti-inflammatory) towards the M1 (mainly pro-inflammatory) microglial phenotype, driven by the persistent presence of amyloid-β plaques, may contribute to these detrimental outcomes (134). Given that a similar inflammatory process can produce different, even opposing, effects depending on the stage of the disease, neuroinflammation has been described as a “double-edged sword”.

Neuroinflammation can arise in response to processes associated to neurodegeneration, CVD and other conditions. For instance, chronic hypoperfusion and hypoxia, which lead to BBB disruption and neurovascular unit impairment, initiate neuroinflammatory responses that further compromise BBB and CNS cells, serving as key contributors to CVD progression and consequently increasing the risk of VCI (135). Additionally, neuroinflammation may impair glymphatic function, leading to amyloid-β accumulation, which promotes neurodegeneration. This, in turn, perpetuates further inflammatory responses, creating positive feedback loops and vicious cycles.

Ageing

As already introduced, many of the discussed pathways, such as those associated with glymphatic dysfunction are impaired either by processes associated with age or by age-related conditions. This provides a plausible explanation to the frequent co-pathology phenomena in diseases impairing cognition, but also a potential explanation of why age is the main risk factor for both neurodegenerative diseases and CVD.

Importantly, it is now clear that people's biological age may differ from their chronological age. Biological age can be estimated through omics profiling. While research on ageing and longevity has predominantly focused on genomics, proteomics is an emerging field that may offer particularly valuable insights in this context. Since many proteins undergo age-related changes, studying an individual's proteome through the analysis of proteins in plasma that originate from specific organs allows for the measurement of organ-specific aging. This approach has facilitated the development of proteomic aging clocks (136), which have demonstrated strong predictive power for all-cause mortality and various age-related diseases, including diabetes and other cardiovascular diseases, AD and PD (137), across ethnically diverse populations. Interestingly, proteomics-constructed biological ageing clocks can predict AD progression independently of, and with comparable strength to, pTau-181 levels (138).

Genetics

Along with age, genetics is one of the most significant determinants of both AD and cardiovascular diseases, including CVD.

ApoE is one of the best examples. ApoE is a protein involved in lipid metabolism and in the amyloid clearance of the brain.

The most common variant in the general population is having 2 copies of the ApoE ε3 allele (ApoE ε3/ ε3) and it is considered to have a neutral risk of AD development. While the ε2 allele seems to have a protective effect for developing AD, having one copy of the ε4 allele increases the risk of AD by approximately 2-3 times, and having two copies of the ε4 allele is currently considered the most frequent form of genetic AD (139). Further, the ApoE ε4 allele is also a key genetic risk factor for the development of a very specific type of CVD: CAA (20,23). The prevalence of ApoE ε4 carriership status (at least one ε4 allele) in a large cohort (the National Alzheimer's Coordinating Centre (NACC)) of individuals with high or intermediate AD neuropathological changes was 52.1% (139) (140), 22% of which (11.4% of the total) were homozygous cases (ε4/ ε4) (22). In contrast, and according to a large UK Biobank Data cohort, the prevalence of ApoE ε4 carriership status in the general population, is 26.5% and, the prevalence of homozygous individuals (ε4/ ε4) is 2.3% (141).

Genome wide association studies (GWAS) have facilitated new insights of genetic risk of dementia. For instance, it has allowed the identification of BIN1, which is the is the most significant genetic risk factor for late-onset AD (LOAD) after the ApoE ε4 allele. Many other genes have also been implicated in AD risk, and like ApoE, some (e.g.,, the ATP5H/KCTD2locus) have been associated both with AD and CVD (142).

Genetic variability may mediate the impact in the individual of the environmental hazards and neuropathological processes (143). In other words, they shape the resilience and resistance of individuals to neurodegenerative and neurovascular changes.

Interestingly, epigenetics -which examines how environmental factors affect gene expression without altering the DNA sequence- serves as a critical intersection between the genome and the exposome, linking external exposures to long-term health outcomes. Epigenetic changes are also valuable to analyse changes posed by ageing.

Finally, genetics and age interact, as illustrated by the age-dependent effects of ApoE. For example, heterozygous carriers of APOE ε4 typically have their first symptoms around 65 years of biological age.

Environmental factors

There is now compelling evidence that the external environment significantly shapes health outcomes of individuals throughout the lifetime. From a health perspective, the "environment" is a wide concept that has been defined as all the "non-genetic external factors" that influence health (144). Thus, it is comprised by a wide range of exogenous physical and non-physical factors that can be broadly divided in:

- Environmental determinants of health, which are the biogeochemical factors external to an individual influencing his health, excluding genetics and the social determinants of health. These include AP, climate change, loss of biodiversity and other processes that could be encompassed under the broad term: global environmental change.
- Social determinants of health, which mainly consist in the social (including cultural factors, values and beliefs, household income, education) and physical (e.g. housing, infrastructure) environment and access to healthcare.

These exogenous factors interact with endogenous factors, such as the genome and epigenetics, microbiome, and metabolic factor to shape individuals' behaviour and psychological outcomes, such as "lifestyle" choices (physical exercise, consumption of harmful products for health like tobacco, alcohol or certain types of food) and stress. Altogether, these factors have a great impact on the health of individuals and populations.

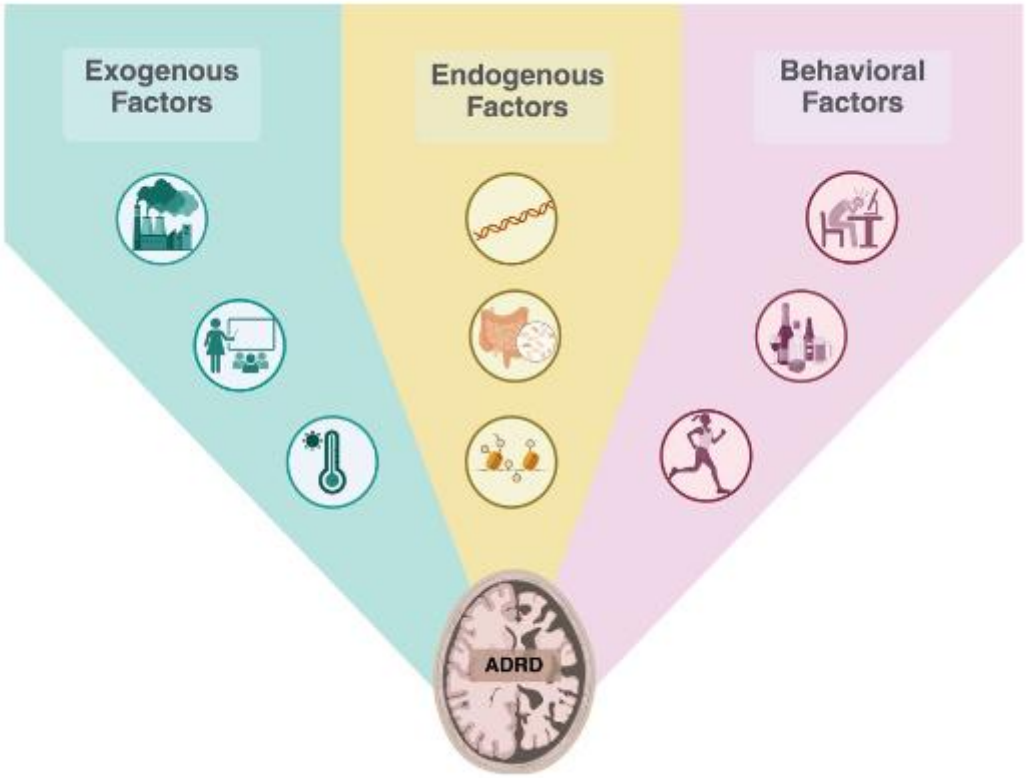
Moreover, the relationship between the environment and health is bidirectional (145–147). AP perfectly exemplifies this environmental-health nexus, as it represents both an environmental and a health issue affecting most of the world's population (148).

All these factors are integrated in the exposome, which is a comprehensive framework that integrates all external factors an individual is exposed to and the biological changes that result from these exposures, mediated by individual susceptibility factors.

However, measuring the exposome is highly complex due to the multiplicity of factors of different types (e.g., biological, socio-behavioural), often interlinked, that must be considered. Some of the necessary data might be sensible or difficult to access, and some of the hazardous factors are particularly difficult to measure accurately, such as individual exposure to AP or psychosocial stress. Their behaviour over time is another challenging point, including single past exposures, continuing exposures or those with lag effects, and the understanding that there are critical time-windows for exposures (e.g., the effects to a hazard may be very different depending on the age of the exposed individual). Additionally, the outcomes might be difficult to assess as well, such as mild cognitive changes. Moreover, studies are usually conducted in a specific moment in a specific population, limiting their applicability to other times and settings.

These concepts can and have been applied to the nervous system, as reflected by the emergence of the concept of "neural exposome". The neural exposome (Figure 7) has been defined as the "internal and external exposures that affect brain and nervous system health over the lifespan of an individual's life". It has been suggested as a potentially valuable framework to investigate risk of AD in a comprehensive manner (149).

Figure 7. The neural exposome for AD and related dementias (ADRD) (extracted from Granov et al. (149))



Since exposure to certain environmental hazards increases the risk for both CVD and AD, this may be one mechanism contributing to the observed association between CVD and AD. AP is a notable example.

Air pollution

Air is considered to be polluted when it contains any extraneous constituent in sufficient quantities to adversely affect the environment or the health of people exposed to it (150).

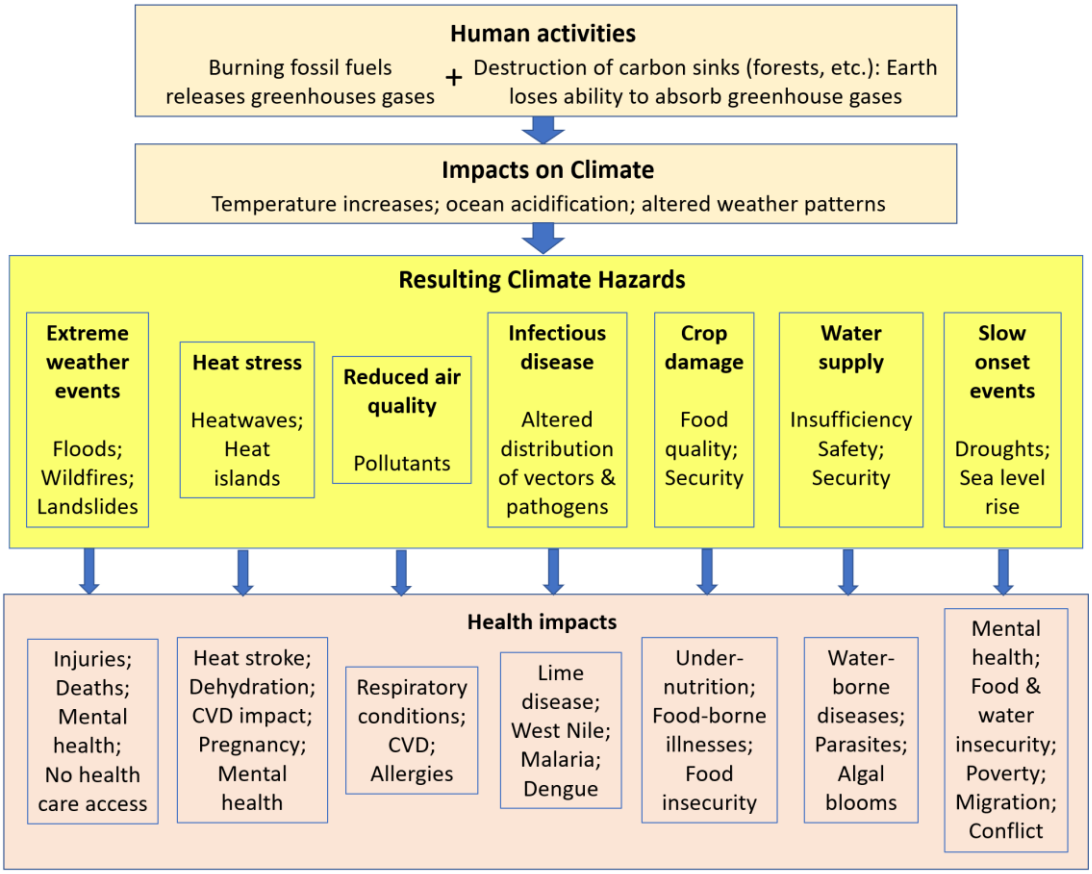
Air pollutants (APs) comprise both gases- such as carbon monoxide, sulphur dioxide and nitric oxides (NO_x) (a collective term that includes nitrogen monoxide, nitrogen dioxide

(NO2) and other nitrogen oxides)- and a heterogeneous group of volatile particles named particulate matter (PM). The latter are classified according to their size. Broadly, it is distinguished between coarse particles or PM_{10} and fine particles or $PM_{2.5}$, which are terms used to designate particles whose diameter is smaller than 10 microns or 2.5m microns correspondingly. The most harmful to health are the finest ($PM_{2.5}$ and ultrafine particles). Coarse ones are mainly composed by dust and soil, whereas the finest ones are generated through combustion of combustible fossils. The term PM_{coarse} is used to refer to particulate matter with an aerodynamic diameter between 2.5 μm and 10 μm - that is, particles larger than $PM_{2.5}$ but smaller than or equal to PM_{10}. $PM_{2.5absorbance}$ is the term used to refer to the approximate quantification of black carbon, one of the most hazardous components of $PM_{2.5}$ both to environment and health.

Combustion of fossil fuels - used for road transport, energy and manufacturing industries, among others- is the main anthropogenic source of outdoor AP. Additionally, indoor AP, mostly from heating and cooking using biomass, places a big burden of disease in populations of lower income countries.

The combustion of fossil fuels is also the main source of emission of greenhouse gases, which are the main cause of climate change. Climate change affects natural and human systems at different levels, including health, through many different pathways, such as through the increase in the number and intensity of extreme weather events, increased food insecurity and lack in the access to safe water and other natural resources, rise and expansion of vector-borne diseases, and increased risk of emerging zoonoses and pandemics (151). These interrelationships between climate change and health are illustrated in Figure 8, which builds on the DPSEEA framework developed by the World Health Organization. Importantly, APs as such have other degrading effects on the environment, such as acid rain, soil degradation and eutrophication (152,153).

Figure 8. DPSEEA framework as applied to health and climate change (extracted from Donovan et al. (24))



AP is considered among the major environmental causes of disease worldwide, and it is estimated that exposure to it causes 7 million premature deaths every year worldwide. Ninety percent of the world's population is exposed to AP over recommended levels (25), so even small effects on health would have huge impacts given the amount of people exposed. While this is particularly severe in urban settings of developing regions, most of the European Union (EU) cities also have AP levels over those recommended by the WHO (25). It may produce adverse health outcomes through a short-term exposure (e.g. peaks of AP have been associated with higher deaths from cardiovascular origin) and long-term exposure. With regards to the latter, AP has been associated mostly with cardiovascular and respiratory diseases, but also with many other adverse health

outcomes, including developmental issues in children and dementia and CVD in adults (26,154). Concretely, it has been estimated that PM contributes to approximately one third of the global stroke burden and about one fifth to the global burden of dementia (26). However, the studies are heterogeneous both in results and in methodological aspects. In fact, the most recent Integrated Science Assessment on $PM_{2.5}$ by the US Environmental Protection Agency, published in 2019 (155), concluded that, overall, the association between exposure to $PM_{2.5}$ and stroke remains inconsistent. Furthermore, to the best of our knowledge, studies exploring the effect of AP on cCVD progression are lacking (156)..

A deeper understanding of the mechanisms linking AP to CVD is needed (157). Several potential mechanisms have been described, including –but not limited to- prothrombotic (158) and proinflammatory effects, increased oxidative stress (157), autonomic dysregulation, endothelial dysfunction that might lead to altered blood pressure and cerebral blood flow (159,159), and neurovascular unit impairment (160), which is essential for maintaining brain health. AP has also been linked to the development of AD lesions, as reflected by the association between exposure to PM2.5 and a higher CERAD score in brain tissue donors (161).

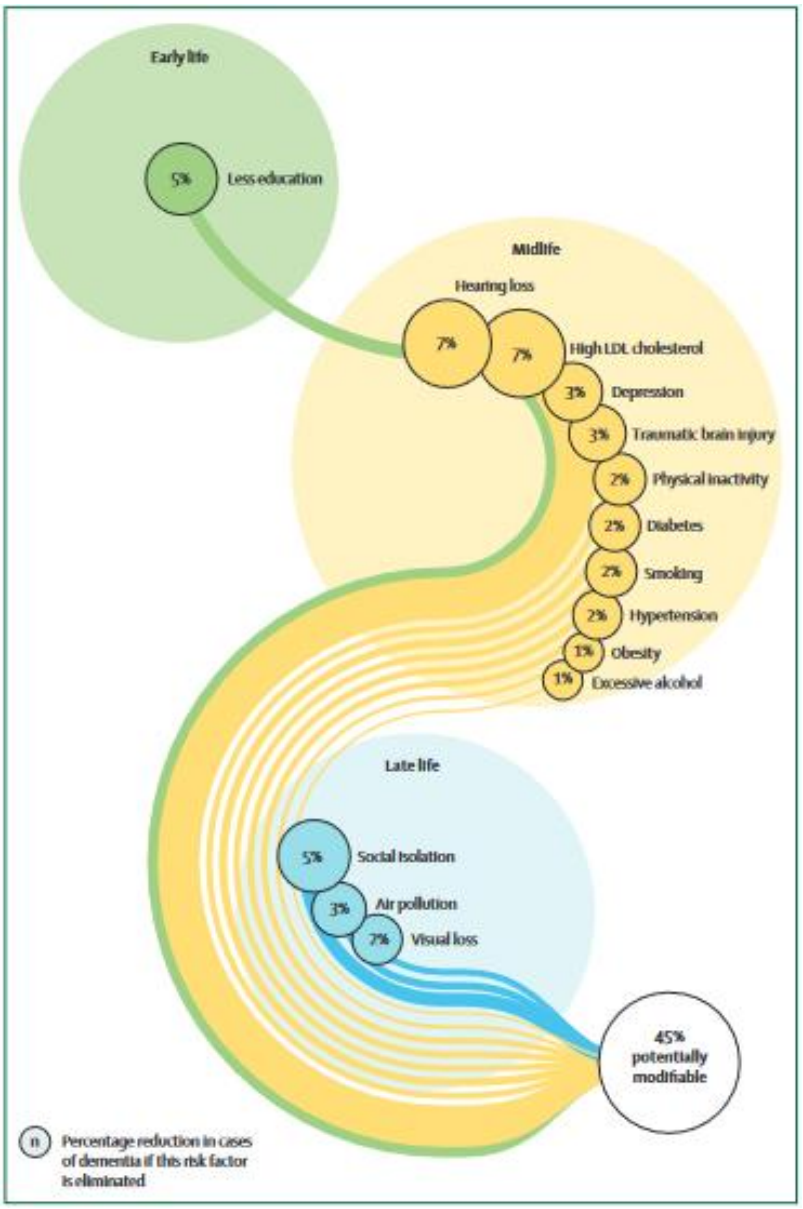
Prevention

Given the substantial impact that diseases affecting cognition, such as AD and CVD, have at various levels -social, economic, and healthcare- it is essential to prioritize the development and implementation of primary and secondary preventive strategies. This refers to the fact that efforts should not only focus on reducing the incidence of these conditions but also on mitigating their effects and improving the prognosis of those already affected by these conditions. It is estimated that up to 50% of dementia cases attributable to any cause could be prevented or delayed through the intervention in modifiable risk factors. Furthermore, early preventive interventions have the potential to significantly reduce the incidence of dementia in the coming years by millions (5,15,162), and thus to significantly diminish the substantial impact that diseases affecting cognition, such as AD and CVD, have at various levels. Importantly, the window for preventive actions is large, since both neurodegenerative diseases and CVD have a long clinical course. The shift towards a biological diagnosis of AD enables to further widen this window through making a prompt and accurate diagnosis. The feasibility of doing so widely has increased with the implementation of blood-based AD BMs in several high-income countries, including Spain. This will enable to implement preventive and therapeutic strategies targeted at high-risk populations (e.g. those with positive AD BMs) and to monitor the effectiveness of whole population interventions (regardless of the individual risk of their constituents).

The 2024 report by the Lancet Commission (162) reviewed the existing evidence on several potentially modifiable dementia risk factors and proposed a model (figure 9) categorizing fourteen of these factors by the stage of life during which they exert their influence. These factors include low education, cognitive inactivity, hearing and vision loss, untreated midlife depression, TBI, physical inactivity, smoking, alcohol consumption, obesity, hypertension after age 40, diabetes, elevated LDL cholesterol in

midlife, social isolation, and AP. The report also calculated the population attributable fraction (PAF) for each risk factor, with low education (5%, early life), hearing loss and high LDL cholesterol (7%, midlife), and social isolation (5%, late life) showing the highest percentages. Notably, some of these risk factors are traditional CVRF, which are the most common contributors to vascular and cardiac diseases in the general population. As previously noted, these conditions are, in turn, the leading causes of CVD (27). The report argues that addressing these fourteen risk factors could prevent or delay up to half of future dementia cases.

Figure 9. PAF of potentially modifiable factors for dementia (extracted from the 2024 Lancet report on dementia prevention (162))



Acknowledging their importance, several specific recommendations for dementia prevention have already been incorporated into clinical guidelines, including the 2019 WHO guidelines on risk reduction for cognitive decline and dementia (163) and the European Academy of Neurology guideline on medical management issues in dementia (164). These guidelines provide varying levels of recommendation strength, with the strongest support for a healthy diet, smoking cessation, and physical activity. In 2017, the American Heart Association/American Stroke Association (AHA/ASA) defined "optimal brain health," which includes maintaining several parameters associated with reduced vascular risk, such as blood pressure, glucose levels, cholesterol, physical activity, body mass index, and diet (165).

With respect to preventive measures in AD-CVD in particular**,** the evidence base remains even more limited. Studies specifically focused on assessing risk factors and potential preventive strategies for this condition are lacking. Until further research is conducted, it may be reasonable to apply recommendations developed for AD and/or VCI.

Preventive strategies and policies can be implemented at various levels and across multiple sectors. For example, diminishing AP may entail investments and/or interventions in public transport and related infrastructure, and the energy and industry sectors. Other examples of actions requiring multisectoral coordination to improve cognition and/or prevent cognitive impairment are ensuring access to quality education and sustained social and intellectual stimulation throughout the life course, lowering smoking rates and improving the management of traditional cardiovascular risk factors, adherence to road safety measures- such as wearing helmets to prevent TBI- as well as addressing depression and other psychiatric conditions (5). Another example is the effective prohibition of certain pesticides that have been associated with PD and AD in several countries in the European region (166,167).

However, involving and coordinating non-health sector actors, each with their own interests and agendas, may present significant challenges.

Prevention in AP

Given the growing body of evidence linking AP with adverse health outcomes, and the huge share of the world population that is exposed to it, AP can be framed as a major public and global health concern. Importantly, exposure to AP is largely preventable through measures such as reducing urban road traffic.

While recommendations are valuable, specific actions, strategies and policies are needed to improve health and environmental outcomes. Indeed, given the heath-environmental nexus, policies aimed at diminishing AP have the potential of having large direct and indirect health (over and beyond diseases affecting cognition) and non-health co-benefits (the additional positive effects that health-related actions or policies have beyond their primary health goals), including serving as a mitigating strategy for climate change. For instance, measures aimed at reducing AP, such as reducing urban road traffic, in addition to reducing exposure to AP would promote more active modes of transport and lifestyle. These, in turn, have well-known benefits in mental health more broadly and at the cardiovascular level.

AIMS AND OBJECTIVES

This project has two overarching aims.

The first overarching aim of this study is to contribute to the body of knowledge of AD-CVD by accurately describing the characteristics of patients with AD-CVD as compared to AD patients without CVD.

To achieve such aim, our specific objectives were:

1. To characterize the clinical, neuropsychological (NPS), imaging and biofluid BMs profiles of the entire sample and of the AD and the AD-CVD groups, including associations between the variables.
2. To compare the clinical, NPS, imaging and biofluid BMs features between the AD and the AD-CVD groups.
3. To identify features and/or BMs that may have diagnostic value in differentiating AD from AD-CVD, if any.
4. To identify features that may have prognostic value when dealing with cases of AD and AD-CVD.
5. To describe the frequence of CAA in our cohort and its relationship with the BMs used.

With regards to objectives 2 and 3, we hypothesized that there must be some identifiable and measurable differences between AD and AD-CVD when such groups are carefully selected. In this line, we hypothesize that AD-CVD cases can be reliably distinguished from “pure” AD cases using clinical, NPS, imaging and biofluids BMs.

The second overarching aim is to evaluate the role of AP for both AD and CVD in cognition and in cCVD.

To achieve such aim, we prosecuted the following objectives:

6. To evaluate the association between AP and covert cerebrovascular lesions in a cross-sectional manner and over time, focusing on changes occurred in relation to AP exposure.
7. To explore the association between AP and cognitive function and cognitive status or diagnosis in a prospective, longitudinal way.

Based on previous literature (26,154), we made two hypothesis with regards to objective 6 and 7. First, we hypothesized that greater exposure to AP would be associated with a higher burden of covert cerebrovascular lesions both at baseline and at follow-up. Second, we hypothesized that greater exposure to AP would be associated with poorer cognitive performance.

To address each of these overarching aims, two separate studies were conducted: Study One -addressing the first overarching aim- and Study Two- addressing the second overarching aim. The methods for both studies will be presented first, in sequence (Study One followed by Study Two), followed by their respective results. The results sections will be followed by a discussion divided into two sections. Finally, the overall conclusions will be presented.

METHODS

METHODS STUDY ONE

In this section, the methods of Study One will be described.

Study population

We conducted an observational, longitudinal, prospective study through the generation of a cohort of consecutive patients diagnosed with either AD or AD-CVD. Since a lumbar puncture was comprised among the study procedures, it also has a minimal interventional approach.

Recruitment was conveniently made from patients coming to routine medical visits at the Cognitive Unit of Vall d'Hebron (VH) University Hospital in Barcelona, Spain, between July 2021 and June 2023. The sample size was determined based on the healthcare activity of such unit, which serves approximately 200 new AD patients annually. An initial recruitment target of 200 patients was set, based on the literature and an expected ratio of two-thirds AD cases to one-third AD-CVD cases. This resulted in a projected sample of 140 AD cases and 60 AD-CVD cases. Although the final sample size was smaller than originally planned due to difficulties in patient recruitment, the proportion of AD to AD-CVD individuals was similar to the proportion initially targeted.

Individuals who are evaluated for the first time at such unit usually already have a neuroimaging -usually plain brain computed tomography (CT) - and a basic blood test - including vitamin B12, folic acid, thyroid stimulating hormone and thyroxin. Visits take place in the presence of a reliable informant whenever possible, and basic sociodemographic and clinical data are obtained through a targeted medical interview. This includes family history, which for this study was considered positive in this regard when a first degree relative is affected by dementia (acknowledging that often the etiological diagnosis is not known by participants/patients). A short cognitive screening

test is usually administered, like the Mini-Mental State Examination (MMSE) (168). The MMSE is one of the commonest cognitive screening tests used in clinical practice, and it was intended to serve as a surrogate of more resource and time-consuming formal NPS assessment. It consists of eleven questions and can be administered in five to ten minutes. It was validated in 1974 and seen to correlate well with more extensive cognitive tests, specifically, with the Wechsler Adult Intelligence Scale (WAIS) (168). The scores of the test range from 0 to 30. Although performance can vary with age and educational level – for instance, illiterate individuals may be unable to complete certain tasks despite preserved cognition- scores above 27 are generally consistent with normal cognition, whereas scores below 24 are typically suggestive of dementia. Intermediate scores (24-26) are most commonly associated with MCI.

Screening eligibility criteria

The following criteria were systematically applied to patients attending VH's Cognitive Unit during the recruitment period:

1. Inclusion criteria consisted in: any individual with mild cognitive impairment (MCI) or mild dementia -corresponding to stages 3 or 4 of the Reisberg Global Deterioration Scale (GDS) (169)- in whom there was a clinical suspicion of AD, whether associated or not to significant CVD.

   The GDS is a widely used tool to assess the severity of cognitive decline in the clinical practices. It categorizes cognitive decline in seven stages, ranging from absence of cognitive decline (GDS 1) to severe dementia (GDS 7).

   An example of a mixed AD-CVD case would be an individual with post-stroke cognitive impairment that shows a progressive cognitive impairment affecting predominantly memory.
2. Exclusion criteria at this stage were:

a. Cognitive impairment could be partially or completely attributed to a concurrent medical condition and/or treatment.
b. GDS score of ≥5 at baseline.
c. Presence of moderate or severe renal function impairment (as defined by a calculated glomerular filtration rate below 50), acknowledging that it may influence plasma AD BMs levels (6).
d. Contraindication for performing a lumbar puncture for determining CSF AD BMs (e.g. chronic anticoagulation).

Participation in the study was offered to all the individuals that met screening eligibility criteria. Those who accepted underwent further procedures as part of the protocol of the study, which are specified below.

Study procedures

1. A NPS assessment was conducted in 93 participants to evaluate cognitive functions, including memory, executive function, language and visuospatial abilities. It was conducted by two neuropsychologists (BG and DL) who are part of the research group. A protocol consistent with standard clinical practice at our centre was conducted, which is designed considering time constraints.

   The twelve tests that were conducted in most of the participants are shown in Table 5. The rate of missing values was high due to clinical constraints; specifically, more cognitively impaired individuals tended to have fewer measurements. For the purposes of this study, we included only those tests with less than 50% missing values. Other tests were performed in an individual basis depending on the predominant clinical features exhibited.

Table 5. Neuropsychological tests

Cognitive domain	Test(s) used
Memory	Three items were obtained from the administration of the Spanish Version of the Rey- Auditory Verbal Learning Test (RAVLT) (250). – Total learning capacity, which reflects the value obtained after the fifth and last repetition of the list of words. – Retention index: calculated as the ratio between delayed recall and the fifth and last attempt of the learning curve. – Total recognition.
Attention	Trail Making test (TMT) A (251)
Executive functions	– Backward span test. – Phonemic verbal fluency, which is measured as the number of words beginning with the letter “p” that could be recalled within one minute (Spanish version of fluency tests of COWAT (252)).
Language	– Semantic fluency, measured as the number of words within the semantic category “animals” that could be recalled within one minute. – Boston Naming Test (BNT) (253) – Correct understanding of commands.
Praxis	Imitation of bimanual posture praxis (4 assessed) (254)
Visuoconstructional abilities	Cube test (255)
Visuospatial abilities	Clock test (256)

2. A 3 MRI Tesla was performed in 95 participants of the cohort. In the remaining 26 participants, MRI was not performed for different reasons. In these cases, the vascular contribution was assessed using CT imaging.

   T1, FLAIR, T2, and susceptibility-weighted imaging sequences were obtained. Historical MRIs within the previous two years were allowed in case these sequences were available.

   Qualitative evaluations were conducted by two collaborating neuroradiologists (DF and ER), who were blind to the clinical information, including the results of

the biofluid BMs. Overall, interrater agreement was strong, with a Cohen's kappa coefficient of 1.00 for nominal variables and an intraclass correlation coefficient (ICC), calculated using Cronbach's alpha, of 0.98 (95% CI: 0.95–0.99, $p < 0.001$).

The main radiological assessments are summarized in Table 6. Notably, the burden of CAA lesions was assessed through the CAA-SVD (Charidimou's) score (170).Other findings such as tumours, signs suggestive of normal pressure hydrocephalus, subdural hematoma, and other abnormalities, were also evaluated and reported.

Table 6. Radiological assessment

Vascular parameters: adapted from STRIVE V2 (170)		
Parameters evaluated	**Locations evaluated/Other specifications**	**Severity**
WMHs of presumed vascular origin	Periventricular (PV) Subcortical	Fazekas scale: 0-3 (66). Mild: 0-1 Extensive 2-3
Infarcts and presumed vascular origin lacunae	Deep: basal ganglia, internal capsule, CSO, brainstem. Territorial: ACA, MCA, PCA Cerebelar: SCA, AICA, PICA	Total number of infarcts Number of infarcts in each location: 0, 1 (1 infarct) or 2 (≥2 infarcts).
PVS	Basal ganglia CSO	Doubal scoring system (256), which ranges from 0 to 4 at each location.
CMBs	Lobar: cortical and subcortical Deep: Basal ganglia, internal capsule, external capsule, thalamus, brainstem Cerebellum	Number in each location classified as follows: 0, 1 (0-5) or 2 (>5) (aligned with Charidimou's score).
(spontaneous) ICH	Cortical Subcortical Cortico-subcortical Basal ganglia Brainstem Cerebellum	
SAH or cSS	Location and number of sulci affected	Focal (≤3) or disseminated (≥4)
CAA (31)	Possible or probable according to the Boston Criteria 2.0	Charidimou's score (170): 0-6, with a maximum severity score of 6. Points are awarded as follows 1) CMBs: a) 2-4: 1 point b) ≥5: 2 points 2) cSS: a) Focal: 1 point b) Disseminated: 2 points 3) CSO-PVS: a) ≥20: 1 point 4) WMHs: 1 point If Fazekas 2 (deep) or 3 (deep or PV).
Cerebral atrophy parameters		
Parameters evaluated	**Comments**	
General atrophy	The GCA score developed by Pasquier (257) was used. This visual rating score applies a 0-4 score to 13 cerebral regions.	
MTA	It was evaluated through Scheltens scale (182), which ranges from 0 to 4, with a maximum severity score of 4. For the purposes of the analysis, we made a mean of each hemisphere Scheltens score. Scores exceeding 1.5 were classified as pathological, as proposed in other studies (200).	

WMHs: white matter hyperintensities; PVS: (enlarged) perivascular spaces; CMBs: cerebral microbleeds; CAA: cerebral amyloid angiopathy; SAH: subarachnoid haemorrhage; cSS: cortical superficial siderosis; GCA: global cerebral atrophy; MTA: medial temporal atrophy; ACA: anterior cerebral artery; MCA: middle cerebral artery; PCA: posterior cerebral artery; CSO: centrum semiovale; SCA: superior cerebellar artery; AICA: anterior inferior cerebellar artery; PICA: posterior inferior cerebellar artery; Periventricular: PV.

3. Blood-based and CSF BMs were analysed in all participants of the study except for controls, who only had blood BMs performed.

Details are provided in Table 7.

a. Blood BMs and ApoE genotyping:

In brief, blood BMs included AB40, AB42, AB42/AB40, 181p-tau, t-tau, GFAP, NfL, sTREM2, and ucGMP. Additionally, participants were genotyped for the ApoE gene.

Ten millilitres of blood were drawn from each participant into Vacutainer® tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ, USA). The samples were centrifuged at 1,500×g (relative centrifugal force) for 15 minutes at 4 °C to allow separation of blood components.

Following centrifugation, the buffy coat fraction- rich in leukocytes- was carefully recovered and stored for DNA extraction, to allow for ApoE genotyping.

DNA extraction was manually performed from frozen EDTA blood samples or buffy coat following the standard operating procedure of the Vall d'Hebron Institut de Recerca (VHIR) Biobank Service. The Danagen DNA extraction kit was used.

DNA concentration was measured by spectrophotometry using a NanoDrop ND2000 (Thermo Fisher Scientific). DNA purity was assessed by the absorbance ratios A260/280 and A260/230, considered acceptable when values ranged between 1.7–2.0 and 1.5–2.2, respectively.

Genotyping of the APOE gene was performed by detecting polymorphisms rs429358 (C___3084793_20) and rs7412

(C___904973_10) using TaqMan® DME probe-based genotyping assays (Applied Biosystems). This analysis was carried out at the National Genotyping Center (CeGen), Xenomic Medicine Group of the University of Santiago de Compostela.

Before analysis, samples were re-quantified using the Quant-T™ system (Invitrogen) and automatically normalized to a final concentration of 30 µL of DNA at 2.5 ng/µL to ensure homogeneity in the genotyping assay.

Additionally, a healthy control group with available biofluid BM data was included as a reference group. This group was drawn from the ISSYS (Investigating Silent Strokes in hYpertensiveS) cohort, which has been previously described (171) and which is further described in the Study 2 section. In brief, this cohort comprised individuals aged 50 to 70 years old with hypertension and without cognitive symptoms or a history of cerebrovascular event.

The same plasma BMs were determined in the three groups: AD, AD-CVD and controls.

b. CSF BMs:

CSF BMs consisted exclusively in AD BMs used in clinical practice and included AB42, ptau181, t-tau, ab42/p-tau181 and abeta42/t-tau. A lumbar puncture was performed using a 25-gauge needle to obtain CSF. Eight cubic centimetres (cc) of CSF samples were collected in polypropylene tubes and processed within four hours of collection. The samples were centrifuged for 10 minutes at 1800g at 4°C. A small portion of the sample was reserved for biochemical analysis, including cell count and levels of proteins and glucose.

Table 7. Fluid biomarkers

BM	Measurement*
Blood BMs	
Aβ42	Simoa Aβ40 2.0 kit and Simoa Aβ42 2.0 kit, respectively (Quanterix Corporation, Billerica, MA, USA).
Aβ40	
Aβ42/40	
p-tau181	Simoa pTau-181 Advantage V2 kit (Quanterix Corporation, Billerica, MA, USA)
t-tau	Simoa Tau 2.0 kit (Quanterix Corporation, Billerica, MA, USA)
GFAP	Simoa Neuro 2-Plex B kit (Quanterix Corporation, Billerica, MA, USA).
NfL	
sTREM2	ELISA kit (MBS7269339; MyBioSource, San Diego, CA, USA), distributed by BionovaCientífica S.L. (Barcelona, Spain).
ucGMP	ELISA kit (CBS-EC013789HU; Cusabio, Wuhan, China), distributed by BionovaCientífica S.L. (Barcelona, Spain).
CSF BMs	
Aβ42	Elecsys system (Roche®), which allows for automated quantification via electrochemiluminescence on Roche's Cobas E601 and E602 instruments
p-tau181	
t-tau	
Aβ42/p-tau181	
Aβ42/t-tau	

Aβ: Amyloid beta; ptau181: phosphorylated tau at threonine 181; t-tau: total-tau; GFAP: Glial fibrillary acidic protein; Nf: neurofilament light chain; sTREM2: soluble fraction of the Triggering Receptor Expressed on Myeloid cells 2; ucMGP: uncarboxylated Matrix Gla Protein.
**Either the Single Molecule Assay (SIMOA) or Enzyme-Linked Immunosorbent Assay (ELISA) were used. All the samples were collected using EDTA (Ethylenediaminetetraacetic Acid)-plasma tubes. All analyses were performed in the biochemistry laboratory of Vall d'Hebron Hospital.*

4. A clinical follow-up was conducted for all the participants, so they were all visited at least twice. The clinical follow-up focused on identifying changes in cognitive impairment and functional capacity, as well as assessing behavioural issues or other significant incidents, including stroke. MMSE test was administered again in most follow-up visits. A focus was put on conversion to dementia, which was

defined as a change from a GDS score of 3, corresponding to MCI, to GDS score of 4 or higher, corresponding to dementia.

Enrolment criteria and diagnosis adjudication:

All previously mentioned procedures were reviewed by two clinical neurologists with expertise in memory clinics (AB and PD), with support of the two collaborating neuroradiologist (ER and DF).

Participants were classified into those with evidence of AD pathology, as indicated by positive CSF AD BMs, and those whose CSF AD BMs were either inconclusive or negative. As shown in the study flowchart in Figure 12 (results section), the positive CSF AD BMs were imperative for including definitively the patients in the study; in other words, they were the main enrolment criterion. We defined positive AD BMs as decreased Aβ1–42 together with increased t-tau or p-tau181 in CSF (53). Notably, the study was conducted prior to the publication and widespread implementation of plasma BMs and before the publication of the 2024 revised NIA-AA criteria for diagnosis and staging of AD (172). As explained in the introduction, these criteria consider that the only AD biofluid BMs that have a diagnosis usage are p-tau181/Aβ42, t-tau/Aβ42, Aβ42/40 (172). However, we confirmed that all individuals classified as having AD-positive BMs under our criteria would still meet the criteria for AD positivity under the revised guidelines.

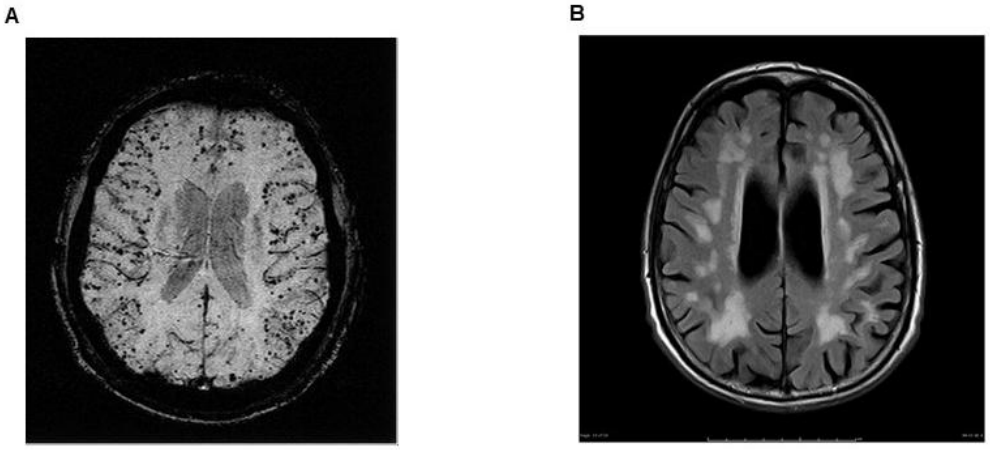
As mentioned, individuals with positive AD BMs were enrolled in the subsequent phases of the study and classified into “pure AD” or AD-CVD after checking once again that no other condition explained their clinical presentation and reviewing the other above-mentioned exclusion criteria.

A participant was classified as AD-CVD rather than AD when at least one of the following criteria was met:

1. A history of stroke or TIA with temporally related onset of cognitive symptoms.
2. Fulfilment of Boston 2.0. criteria for probable CAA (36). The patients who met the possible CAA criteria but did not satisfy any other criteria for significant CVD were classified as AD rather than AD-CVD, acknowledging that the specificity of this category for CAA in populations presenting with cognitive symptoms and no history of intracranial haemorrhage (ICH) is low (36).
3. Adherence to VASCOG criteria (31). Specifically, cognitive impairment consistent with a vascular origin, along with at least one of:
    a. One or more large ischaemic infarct/s.
    b. More than two lacunar infarcts (excluding the brainstem and the cerebellum) or one to two if strategic and/or associated with extensive WMHs.
    c. Two or more ICH (>5 mm in its widest diameter, to differentiate from CMBs).
    d. One strategic (e.g., involving the thalamus or other key areas for cognition) ischaemic or haemorrhagic (ICH) infarct.
    e. Extensive and confluent WMHs, defined by a score of 2 or 3 on the Fazekas scale (66).
    f. Combinations of the above criteria (e.g., WMH scores of 2 or 3 combined with one or two lacunar infarcts).

The criteria we used tried to acknowledge the wide spectrum of CVD, including non-symptomatic or very mild presentations which may go unnoticed and undiagnosed until they lead to VCI (47). These include cCVD, which, as discussed in the introduction, refers to cerebrovascular lesions that occur without provoking any acute episode.

Figure 10. One illustrative example of CAA with multiple lobar CMBs(A) (189) and one illustrative example of subcortical WHMs (B) (42)



Statistical analysis

Both descriptive and inference statistics were performed using version 22 of the SPSS program or R software.

The cohort was described, comparisons between the AD and the AD-CVD made, and existing associations among clinical and all type of BMs explored, including each participant's Charidimou's score (170). Furthermore, we performed a sensitivity analysis consisting in comparing the AD and the AD-CVD groups after excluding participants who met no other criteria for "significant CVD" aside from extensive white matter affection. This exclusion was based on the recognition that WMHs is a sign of neurovascular unit dysfunction, which may result from neurodegenerative processes alone (117).

The normality check was conducted using formal statistical tests, including the Shapiro–Wilk and Kolmogorov–Smirnov tests. Depending on the data distribution, t-tests or Mann–Whitney U tests were used for continuous variables, and chi-square tests for categorical variables. The Spearman test was used for analysing associations between quantitative variables. Ordinal qualitative variables with more than five categories were

treated as quantitative variables. Correlation strength was categorized as weak (0.1-0.4), moderate (0.4-0.7), or strong (>0.7), and associations were classified as either positive or negative.

For biofluid BMs and NPS variables that showed significant differences between groups, we explored optimal cutoff points for distinguishing AD from AD-CVD using receiver operating characteristic (ROC) curves derived from logistic regression models. Variables such as age, sex, years of education and ApoE carriership status were included in the models when appropriate. An area under the curve (AUC) value below 0.7 was deemed inadequate for reliable discrimination between groups.

We accounted for multiple comparisons by applying a false discovery rate (FDR) correction. Only statistically significant correlations (as defined by p-value<0.05) after such correction were considered. Clinical plausibility and meaningfulness were considered throughout the analysis.

NPS data analysis

Given the limited number of tests available, we opted to analyse each individual test rather than attempting to construct composite scores or cognitive domains for the analysis.

Acknowledging that age, sex, and years of education affect NPS data, we eliminated their potential effect through calculating the residuals obtained from multiple linear regression models in which the independent variables were these three variables, and the dependent variable was each respective NPS test score. Since we did not have the exact number of years of education for all participants (specifically, for 40 individuals), but rather a categorical score ranging from 0 to 4 (0 = no education, 1 = primary school, 2 = secondary school, 3 = college), we opted to approximate the years of education for these participants as follows: 8 years for primary school, 12 years for secondary school, and 16 years for college.

Since in the literature there are some, but conflicting, findings suggesting that ApoE status alone is associated with worse cognitive performance in middle-aged and elderly individuals (173–175), we ultimately included the ApoE4 carriership status variable in the regression models. However, we did not find any significant interaction between ApoE carriership status and the NPS variables in these models.

Biofluid BMs analysis

All BMs variables exhibited very asymmetric (non-normal) distributions, so logarithmic transformations were applied to facilitate their analysis.

Due to variations in CSF Aβ-42 and related ratio cut-off values over the course of the study- attributable to changes in collection tubes- we adjusted these values using a correction coefficient. This coefficient was calculated by dividing the mean Aβ-42 value for each tube generation by the overall mean of the two generations. Since cut-off values for p-Tau and t-Tau remained consistent across tube generations, no correction was applied to these BMs.

Plasma BMs were analysed in two different batches. To assess consistency across the two batches, some individuals' samples were reanalysed in the second batch, allowing us to compute the coefficient of variation (CV) for each. All plasma BMs exhibited a CV below 20%, except for GFAP and NfL. To correct for batch effects in these two variables, we applied a correction factor calculated by dividing the mean value of each batch by the overall mean across both batches.

We performed interaction analyses to evaluate the potential effects of age, sex and ApoE4 carriership status on plasma and CSF BM levels. No significant interactions were found between these variables (age and sex and ApoE4 carriership status) and any of the biofluid BMs. Specifically, the variable inflating factor (VIF) for all covariates was approximately 1 and remained below 2 in all cases, indicating minimal multicollinearity.

Ethics

VH Ethics Committee reviewed and approved all aspects of the study protocol before any procedure was performed (PR (AG) 243/2019).

The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

An informed consent, which included a copy for the patient and/or the primary caregiver, was obtained and signed, prior to the realization of any of the procedures specifically related to the study. All the implications of participating in the study were explained in detail.

Each participant was assigned a code to anonymize personal information.

All lumbar punctures were performed following standard clinical practice and following stablished recommendations (176). Participants were invited to join the study at the time CSF obtention for core AD BMs analysis was proposed to achieve a diagnosis. Therefore, the procedure would have been offered to all included patients regardless of their participation in the study.

Regardless of their final participation status, all participants received recommendations and information regarding their condition, along with tailored guidance and specific support depending on their individual circumstances.

METHODS STUDY TWO

In this section, the methods of Study Two will be described.

The ISSYS study

The study 2 was nested within the ISSYS (Investigating Silent Strokes in hYpertensives Study) cohort -the same cohort from which the control group of study 1 was drawn. The ISSYS cohort was established to determine the prevalence and progression of cCVD and cognitive impairment, both at baseline and after four years of follow-up, in a group of hypertensive individuals aged 50 to 70 years old, with no prior history of stroke or dementia.

Participants were recruited from one of the fourteen primary care centres in the northern area of Barcelona, Spain.

The sample size was calculated considering the prevalence of CBI reported in previous studies (177). Specifically, it was calculated to detect a CBI prevalence of approximately 10%, with a 95% confidence interval and a 2% margin of error. The resulting required sample size was n=865; however, an additional 20% of participants were included to account for potential losses during follow-up, yielding a final sample size of 1037 individuals.

A random, stratified sampling procedure was used, with stratification based on the prevalence of hypertension by age and sex. Individuals were invited to participate telephonically.

Enrolment visits were conducted between 2010 and 2012, and all participants provided written informed consent.

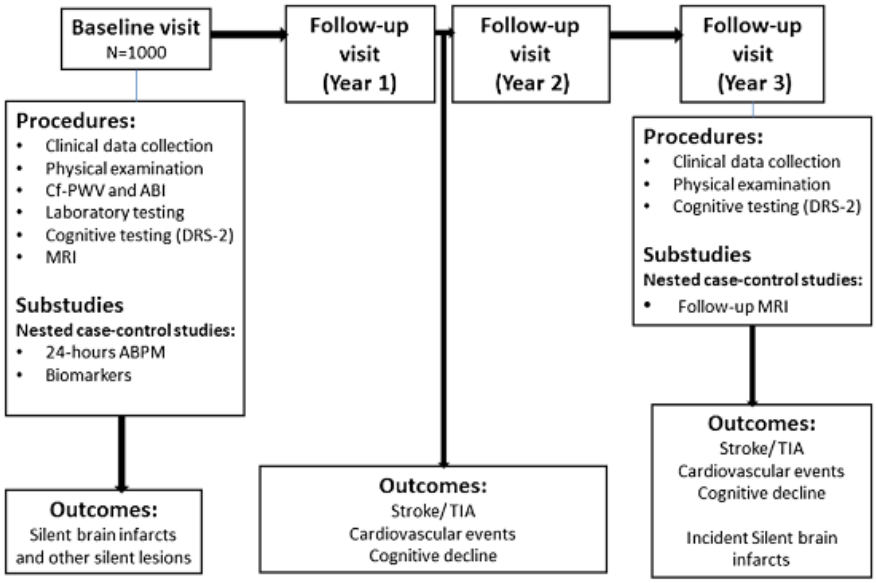
Inclusion criteria included being between 50 and 70 years old and having had a diagnosis of essential hypertension diagnosis for at least one year.

Exclusion criteria included a prior diagnosis of stroke or dementia, or any contraindication to undergo a cerebral MRI, and having a serious or potentially life-threatening condition at the time of enrolment.

All participants attended a baseline visit, during which demographic (e.g., age, gender, ethnicity, current and former occupation, maximum educational level), clinical (e.g., CVRF, medications) and anthropometric data were collected. Global cardiovascular risk was estimated using the REGICOR (Registre GIroní del COR) score (178), an adaptation of the Framingham score (179), which, in turn, is a measure of global cardiovascular risk. The total cardiovascular risk is classified according to the score obtained in REGICOR in low (<5%), moderate (5-9.9%), high (10-14.9%) and very high (≥15%). Blood pressure was measured at the baseline visit and through ambulatory blood pressure monitoring. All participants were assessed for hypertension-mediated organ damage beyond the brain. This assessment included an electrocardiogram, carotid-femoral pulse wave velocity (cf_PWV) -a marker of arterial stiffness- and the ankle-brachial index (ABI). More details are provided in the published protocol by Riba-Llena et al. (28).

A schematic representation of the study procedures is provided in Figure 11 and are further detailed below. Notably, all procedures were conducted and all the data gathered before the main author of this work joined the research group.

Figure 11. ISSYS procedures and outcomes (extracted from Riba-Llena et al. ((28))



Cognitive assessment (within the ISSYS study)

The same procedure was used to evaluate cognitive status at baseline and at follow-up (four years after).

Cognitive screening was first conducted using the Dementia Rating Scale, second version (DRS-2) by Mattis, a tool validated in the Spanish population (38). The DRS-2 includes five subscales: attention (0–37 points), initiation/ perseveration (0–37 points), construction (0–6 points), conceptualization (0–39 points), and memory (0–25 points), with a total score ranging from 0 to 144 points.

To assess executive function, we calculated a composite score by averaging the conceptualization and initiation/perseveration subscale scores. Raw DRS-2 scores were standardized into Z-scores (individual score minus sample mean, divided by the sample SD) at each time point to normalize the results. Global cognitive function for the full cohort was estimated using the Z-score of the total DRS-2 score, adjusted for age and education.

Participants who scored ≤8 on the age- and education- adjusted DRS-2 were considered as possibly cognitively impaired and were invited for an extensive clinical visit by a neurologist and for a NPS assessment by a specialised neuropsychologist to establish a cognitive status diagnosis (i.e. normal aging (NA) vs MCI vs dementia) (38). The NPS tests used in this evaluation are summarized in Table 8.

MCI diagnosis was assigned by consensus between a neurologist and the neuropsychologist for individuals with:

- A self- or informant-reported, acquired cognitive complaint in at least one domain,
- Onset dating back from months to years,
- Objective cognitive impairment (i.e. performance below 1.5 SD from age- and education- adjusted normative means), and
- Preserved or only minimally impaired activities of daily living.

Participants who scored above the DRS-2 cognitive impairment cutoff and did not meet criteria for MCI were classified as NA. Thus, all participants were categorized as either NA or MCI at both baseline and follow-up.

Based on changes in cognitive status from the baseline visit to the follow-up visit, participants were assigned to one of the following cognitive transitions:

1. Stable NA: NA at both baseline and follow-up.
2. Reversion to normal: MCI at baseline and NA at follow-up.
3. Stable MCI: MCI at baseline and follow-up.
4. Incident MCI: NA at baseline and MCI at follow-up.

Table 8. NPS evaluation (modified from (201))

Cognitive test	Function evaluated
Rey Auditory Verbal Learning Test (250)	Immediate and delayed verbal memory
Visual reproduction I and II of WMS-III (258)	Immediate and delayed visual memory
TMT A and B (251)	Attention and task switching
Block design of WAIS-III (250)	Perception (spatial component)
Fluency tests of COWAT (252)	Language and executive function
Stroop test (259)	Selective attention, cognitive flexibility and processing speed
Clock drawing (250)	Visuospatial and constructional abilities
Symbolic gesture of Barcelona's test (254)	Gestural praxis
Sequences and imitation of postures of Barcelona's test (254)	Ideomotor praxis

Neuroimaging assessment (within the ISSYS study)

All participants underwent a baseline 1.5. Tesla cerebral MRI. MRI sequences included T1-weighted, FLAIR, T2-weighted and gradient echo images.

As shown in the study flowchart in the Results section, a second cerebral MRI was performed after the third year of follow-up to detect changes and assess the incidence of new cerebrovascular lesions. Due to budget limitations, no tall participants were invited for the follow-up MRI. Participants who already had cerebrovascular lesions on the baseline MRI were prioritized.

The main lesions evaluated at both baseline and follow-up are listed in Table 9. Given the low incidence of new CBI and CMBs, all lesions were grouped into a combined progression score. Radiological progression was defined as the sum (1 point each) of the four primary radiological markers of CVD: incident CBI, incident CMB, incident PVS, and/or marked progression of WMHs.

All MRI were independently reviewed by two neuroradiologists, blinded both to clinical data and the time point of the scan. Discrepancies were resolved through discussion between the two readers or by consensus with a senior researcher.

Table 9. Radiological assessment

Vascular parameters: adapted from STRIVE V2 (170)		
Parameters evaluated at baseline	**Locations evaluated/Other specifications**	**Severity**
WMHs of presumed vascular origin	Periventricular (PV) Subcortical (SC)	Fazekas scale: 0-3 (66). Mild: 0-1 Extensive 2-3
Infarcts and presumed vascular origin lacunae	Deep: basal ganglia, internal capsule, CSO, brainstem. Territorial: ACA, MCA, PCA Cerebelar: SCA, AICA, PICA CBI were defined as lesions ≥3 mm in diameter in their widest dimension that have cerebrospinal-fluid-like signal features in all pulse sequences and a hyperintense rim surrounding them in FLAIR weighted images.	Total number of infarcts Number of infarcts in each location: 0, 1 (1 infarct) or 2 (≥2 infarcts).
PVS	Basal ganglia CSO Considered as lesions <3cm and without gliosis rim.	Doubal scoring system (250), which ranges from 0 to 4 at each location.
CMBs	Lobar: cortical and subcortical Deep: Basal ganglia, internal capsule, external capsule, thalamus, brainstem Cerebellum	Number in each location
(spontaneous) ICH	Cortical Subcortical Cortico-subcortical Basal ganglia Brainstem Cerebellum	
Parameters evaluated at follow-up	**Locations evaluated/Other specifications**	**Severity**
Incident CBI		
Incident CMBs		
Incident PVS		
WMH progression		Rotterdam scale: progression was considered as "marked" when the score in such scale was >2.5 (260)
Combined progression score	Incident CBI + Incident CMBs + incident PVS + "marked" WHMs progression	The presence of any of the four components provides one point each.

Blood BMs (within the ISSYS study)

Blood samples were collected from all participants using eight tubes: three containing EDTA and one containing citrate anticoagulant. A complete blood count and basic biochemical analyses were performed. Following centrifugation (15 minutes at 3,500 rpm) plasma samples were aliquoted and stored at -80ºC for the analysis of blood BMs.

As previously mentioned, BMs from 28 participants were used as control group for the Study 1.

Exposition to AP

Exposition to AP was estimated by the Barcelona Institute for Global Health using land-use regression (LUR) models and the geocoded residential addresses of the participants. Participants were asked during the follow-up period whether their residence had changed during the study period, and their responses confirmed that it had not. Moreover, 91% of the respondents (n=503) reported not having changed their usual residence for 10 years or more.

The LUR models were based on AP data collected between 2008 and 2011 from monitoring stations located at urban and regional sites (20 sites for PM and 40 sites for NO2). Specifically, the following APs were measured, which are those among which the most harmful effects to health have been documented: NO2, NO_x, PM_{10}, $PM_{2.5}$, PM_{coarse} and $PM_{2.5\ absorbance}$. Annual average concentrations were estimated by accounting for temporal trends using data from continuous reference monitors. Separate models were developed for each air pollutant. These models also included multiple other variables, such as traffic indicators, distance to major roads, and proximity to green spaces, derived from geographical information systems.

The models had been specifically developed for the city of Barcelona as part of the ESCAPE project, which investigates the health effects of long-term exposure to AP in human populations (181).

Statistical analysis

All analyses were conducted with IBM SPSS version 20 (Chicago, IL). Intergroup differences were assessed with the $\chi 2$ test for categorical variables, and the *t*-test, ANOVA, Mann-Whitney U test, or Kruskal-Wallis test for continuous variables, depending on the distribution of the data. Correlations between continuous variables were evaluated using Pearson or Spearman correlation coefficients, as appropriate. A *p*-value<0.05 was considered statistically significant.

Binary logistic regression models were used to evaluate the associations between APs and cerebrovascular lesions (presence/absence) and cognitive outcomes. We also explored associations between APs and various CVRF and related conditions (e.g., AF) through subgroup analyses. Each individual air pollutant was modelled separately. In addition, multipollutant models were constructed and assessed.

Baseline outcomes included the presence of CBIs, extensive PV, or SC WMHs, CMBs and extensive PVS at the basal ganglia or CSO. Follow-up outcomes included incident CBIs, incident CMBs, and progression (yes/no) of SC or PV WMHs and PVS. Additionally, a composite progression score was created to represent the overall burden of radiological progression.

All models included years of education as a proxy for socioeconomic status. Initial models were adjusted for age and sex. For multipollutant models, stepwise selection was applied when appropriate. For vascular outcomes, the REGICOR score was added as a covariate. For cognitive outcomes, baseline cognition and WMH progression were also included in the models.

Odds ratios (OR) and Beta coefficients were used to estimate associations between APs and outcomes, expressed per 10-unit increase in each pollutant, except for PM2.5 abs, in which estimates are expressed per unit increase. 95% confidence intervals were reported for all estimates.

Ethics

The study protocol was approved by the Ethics Committee of VH, as well by the Ethics Committee at IDIAP research institute.

Written consent was obtained for all participants before their inclusion in the study, and after having been fully informed about the study and understood the information.

RESULTS

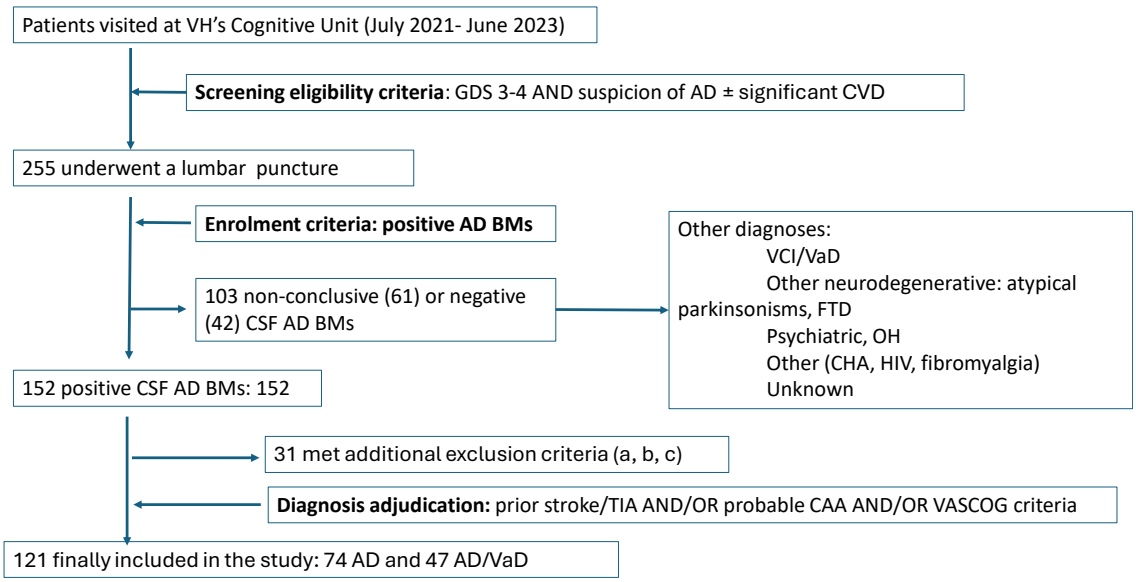
RESULTS STUDY ONE

In this section, the results of the study are presented in accordance with the study objectives, with each subsection addressing a specific objective. The initial subsections are further divided to report the main findings related to demographic and clinical characteristics, NPS tests, and imaging and biofluid BMs (CSF and blood).

Objective 1. Description of the cohort and associations

This subsection presents the main findings related to the characteristics of the full sample, as well as the two diagnostic groups within it (AD and AD-CVD). It also includes the relevant associations among variables identified across the analyses.

Figure 12. Flowchart Study One



As illustrated in the study flowchart (Figure 12), among participants who accomplished screening eligibility criteria and thus underwent a lumbar puncture to obtain CSF samples

for determining AD BMs, we differentiated between those with a completely normal CSF AD BMs profile (negative CSF AD BMs; n=42 individuals) and those with an abnormal AD BMs profile. We further differentiated the latter between those consistent with an AD diagnosis (positive CSF AD BMs; n=121) and those with non-conclusive CSF AD BMs, that is, abnormal levels of either AB-42, pTau or tTau, but with normal AB-42/pTau and AB-42/tTau ratios (n=61 individuals). Participants with a normal CSF AD BM profile were younger than AD patients (72±9.2 vs. 73.3±6.9, p=0.026). There were no statistically significant differences in other demographic and clinical features among these groups, such as sex, years of education, the prevalence of traditional CVRF, the familiar history of cognitive impairment, or the baseline MMSE score (normal 24.5±3.9, non-conclusive 24.6±5.2).

As shown in Figure 12, the final diagnosis of these groups was heterogeneous, including "pure" VCI and primary psychiatric conditions.

121 participants accomplished enrolment criteria, 74 of whom (61%) were classified as AD and 47 of whom (39%) were classified as AD-CVD following the above-specified diagnostic adjudication criteria.

The median interval between baseline and follow-up visits was 12 months (interquartile range (IQR): 10–14.5). The AD-CVD group had a slightly higher median interval of 13.1 months (IQR: 9.5–15) compared to 12 months (IQR: 10–14) in the AD group. However, this difference was not statistically significant (p = 0.21).

Additionally**,** 28 hypertensives individuals without stroke nor cognitive symptoms from the ISSYS cohort (171) were included as a reference control group for the blood BMs analysis, as earlier specified. Their median age was 72 years (65.5-74), 9 (32%) were women, and they had had 9 (6-11) years of education. 50% had never been smokers (10.7% current smokers), 8 individuals (28.6%) also had diabetes mellitus and 17 (60.7%) also had dyslipidaemia. There were no statistically significant differences

between the control group and the AD and AD-CVD groups with regards to sociodemographic variables.

Demographic, clinical, and NPS features (AD and AD-CVD)

Table 10 and Table 11 show the primary characteristics of the main sample (AD-CVD and AD). The former focuses on demographic data and comorbidities, while the latter highlights features related to the clinical presentation of the disease.

Table 10. Demographic characteristics, main comorbidities, and risk factors

Variables		Total: 121	AD: 74 (61%)	AD_CVD: 47 (39%)
Age, years		74.1 (68.5-78-2)	75.2 (70.2-78-4)	77.5 (71.5-79.5)
Sex, women		72 (59.5%)	49(66.2%)	23 (48.9%)
Education	Illiterate	17 (14.16%)	13 (16.6%)	5 (11.9%)
	Primary School	54 (45%)	31 (39.7%)	23 (54.8%)
	High school	27 (22.5%)	18 (23.1%)	9 (21.4%)
	University	21 (17.5%)	16 (20.5%)	5 (11.9%)
	Years	8.0 (6-9)	8.0 (3-9)	8.0 (6-9.5)
Smoking status	Current	13 (10.7%)	10 (13.5%)	3 (6.4%)
	Previous	23 (19.2%)	13 (17.5%)	10 (21.3%)
	Never	83 (68.6%)	50 (67.6%)	33 (70.2%)
Alcohol (usual consumption)		16 (13.2%)	10 (13.5%)	6 (12.8%)
Hypertension		72 (47.1%)	41(55.4%)	32 (76.2%)
Dyslipidaemia		58 (47.9%)	37 (50.0%)	21 (50.0%)
Diabetes mellitus		28 (23.1%)	19 (25.7%)	9 (19.15%)
Ischaemic heart disease		9 (7.4%)	5 (6.8%)	4 (8.5%)
Obstructive Sleep Apnoea		10 (8.3%)	5 (6.8%)	5 (14.3%)
Previous stroke	All types	**7 (5.8%)**	**1 (1.4%)**	**6 (12.8%)**
	Cortical	3 (2.5%)	NA	3 (6.4%)
	Lacunar	2 (1.7%)	1 (1.4%)	1 (2.1%)
	Undetermined topography	2 (1.7%)	NA	2 (4.8%)
Epilepsy		4 (3.3%)	3 (4.0%)	1 (2.1%)
Traumatic Brain injury		3 (2.5%)	2 (2.7%)	1 (2.1%)
Kidney disease		5 (4.1%)	3 (4.1%)	2 (4.8%)
Anxiety disorder		13 (10.8%)	7 (9%)	6 (14.3%)
Mood disorder		28 (23.3%)	20 (25.6%)	8 (19%)
Hearing loss		13 (10.7%)	7 (9.5%)	6 (12.8%)
ApoE status (n=115)	ε3/ε2	3 (2.6 %)	1 (1.3%)	2(4.3%)
	ε3/ε3	47 (40.9%)	28 (37.8%)	19 (40.4%)
	ε4/ε2	4 (3.5%)	4 (5.4%)	0
	ε4/ε3	51 (44.4%)	36 (48.7%)	15 (31.9%)
	ε4/ε4	**10 (8.7%)**	**2 (2.7%)**	**8 (17.0%)**

Data are displayed as median (IQR), or number of cases (%) as appropriate
Bold characters display statistically significant differences (p<0.05).
NA: Non-appliable

As shown in table 10, the median age of the participants at baseline was 74 (IQR: 68.5-78-2) years, with a majority being women (59.5%). The most prevalent CVRF were hypertension and dyslipidaemia, each present in 47% of the participants. 30% of the participants were either current or previous smokers. Other common medical antecedents included a history of mood disorders (23.3%). No patient had an AF, as usually anticoagulation is prescribed for this condition, and it was deemed inappropriate to withdraw anticoagulant therapy for performing a lumbar punction. None had peripheral arterial disease. Although 4 patients had chronic kidney failure, none of them had a calculated glomerular filtration rate (GFR) under 50%.

We obtained ApoE genotyping in 115 individuals. The most prevalent genotype was ε4/ε3 (51 individuals, 44.3%). 65 individuals (53.7%) had at least one ε4 allele (ApoE ε4 carriership status). ApoE ε4 carriership status was higher among those individuals who had a positive family history of dementia (71%) and among those classified as AD-CVD based solely on extensive WMHs. ApoE ε4 carriership status showed a weak correlation with lower scores in the retention index, although it did not show any significant association with any radiologic or biofluid BM.

Table 11. Main clinical features of the participants

Variables		Total=121	AD (n=74)	AD_CVD (n=47)
Age at onset, years		72.0 (68-76)	71 (67-75)	73 (68.5-76)
Early-onset (<65y)		21 (17.4%)	14 (18.9%)	7 (14.9%)
Family history* (n=102)		56 (46.3%%)	36 (46.2%)	20 (47.61%)
Family history (of those with early onset)		7 (23.8%)	5 (35.7%)	2 (28.6%)
First symptom	Memory	96 (79.3%)	63 (85.1%)	33 (70.2%)
	Language	9 (7.5%)	5 (6.8%)	3 (6.4%)
	Executive functions	13 (10.8%)	4 (5.4%)	9 (19.1%)
	Visuospatial	1 (0.8%)	1 (1.4%)	1 (2.1%)
	Neuropsychiatric	2 (1.7%)	2 (2.7%)	1 (2.1%)
Anosognosia		31 (25.6%)	16 (21.6%)	15 (31.9%)
Anxiety		34 (28.1%)	22 (29.7%)	12 (25.5%)
Apathy		27 (22.3%)	16 (21.6%)	11 (23.4%)
Mood disorder		44 (36.4%)	28 (32.4%)	16 (34.0%)
Irritability		37 (30.6%)	24 (30.8%)	13 (27.6%)
Psychosis		12 (9.9%)	5 (6.8%)	7 (14.9%)
Delirium		3 (2.5%)	2 (2.7%)	1 (2.1%)
Parkinsonism		10 (8.3%)	4 (5.4%)	6 (12.7%)
MMSE at baseline (n=111)		24.0 (20-26)	24.0 (20-26)	23.5 (20-26)
MMSE at follow-up (n=82)		23(24-25)	23 (20-26)	23.5 (20-26)
GDS at baseline	3 (MCI)	93 (76.9%)	54 (73.0%)	39 (83.0%)
	4 (mild dementia)	28 (23.1%)	20 (27.0%)	8 (17%)
GDS at follow-up**	3 (MCI)	61 (50.4%)	41 (55.4%)	20 (42.5%)
	4 (mild dementia)	49 (40.5%)	26 (35.1%)	23 (49.0%)
	5 (moderate dementia)	4 (3.3%)	4 (5.4%)	0
	6 (moderately severe dementia)	3 (2.5%)	1 (1.3%)	2 (4.3%)
Converted to dementia***	GDS 3 to ≥4	35 (37.6%)	**17 (23%)**	**18 (40%)**
No pharmacologic treatment		9(7.4%)	6 (8.1%)	3 (6.4%)
Anticholinesterase Inhibitors		83 (68.6%)	53 (71.6%)	30 (63.8%)
Memantine		2 (1.7%)	1 (1.3%)	1 (2.4%)
Antidepressants		59 (48.8%)	37 (50%)	23 (48.9%)
Antipsychotics		19 (15.7%)	10 (13.5%)	9 (19.1%)

Data are displayed as median (IQR) or number of cases (%) as appropriate
Bold characters display statistically significant differences (p<0.05).
** Family history was not available for 19 participants.*
*** No patient with GDS 7 (severe dementia) at follow-up.*
****No patient improved their functionality according to GDS. Percentages are based on participants with GDS 3 at baseline.*

Table 11 presents the clinical characteristics of the participants at baseline. Nearly half of the participants reported a family history of dementia. Memory-related symptoms were

the most common clinical presentation. Approximately 20-30% of the participants exhibited neuropsychiatric symptoms at the onset. The median MMSE score at baseline was 24 (IQR: 20-26), which is consistent with an MCI and the GDS at baseline, as 76.9% of participants were rated at stage 3. During the follow-up period, 37.6% of these individuals progressed to dementia. Most participants had been prescribed pharmacologic treatment at enrolment, with the most used medications being anticholinesterase inhibitors (68.6%) and antidepressants (48.8%).

The main differences in clinical features between the AD and AD-CVD groups will be discussed in the subsection addressing Objective 2.

Early-onset patients performed better on the BNT30 compared to late-onset patients ([0.75 [0.3-0.9] vs 0.15 [-0.6-0.58], p=0.01). No other statistically significant differences were found regarding NPS performance. As further detailed in the subsection addressing Objective 4, both baseline and follow-up MMSE and GDS scores were correlated with performance on several NPS tests.

Imaging BMs

Table 12 summarizes the main radiological features of the two main groups within our cohorts. The most frequent cerebrovascular radiological sign was PVS: 90.1% exhibited some degree of PVS in the basal ganglia -which are most related to microangiopathy associated with traditional CVRF such as hypertension- and 45% of the cohort showed some degree of PVS in the CSO -potentially indicative of underlying CAA.

39.7% of the entire cohort exhibited at least one brain infarct, with a higher proportion of these being in deep brain structures. No patients exhibited intracranial haemorrhages.

Between 17 and 27% of participants had extensive WMHs.

Notably, significant MTA, defined as a score of 1.5 or higher on the Scheltens scale (range 0-4) (182), was observed in 52.1 % of the participants. MTA showed a moderate

positive correlation with advancing age (r = 0.40, p = 0.04). Accordingly, late-onset patients had more MTA atrophy than early-onset patients (72.5% vs 33.3%, p=0.08). MTA also exhibited a moderate positive correlation with greater PV WMH severity (r=0.339, p=0.05).

Of the 47 participants in the AD-CVD group, 15 (31.9%) were classified as such based solely on the presence of extensive WMHs.

Table 12. Imaging characteristics

	Total: 121	AD: 74 (61%)	AD_CVD: 47 (39%)	p value	
Vascular					
Infarcts					
All locations	48 (39.7%)	16 (21.6%)	32 (68.1%)	**0.04**	
Deep (1)	11 (9.1%)	5 (6.8%)	6 (12.8%)	**0.02**	
Deep (>1)	14 (11.6%)	5 (6.8%)	11 (23.4%)		
Territorial (1)	4 (3.3%)	1 (1.4%) *	3 (6.4%)	**0.04**	
Territorial (>1)	4 (3.3%)	0	4 (8.5%)		
Cerebellar (1)	9 (7.4%)	4 (5.4%)	5 (10.6%)	>0.05	
Cerebellar (>1)	7 (5.8%)	3 (4.1%)	4 (8.5%)		
WMH (Fazekas≥2) (66)					
Subcortical	21 (17.4%)	**4 (5.4%)**	**17(36.2%)**	**<0.001**	
Periventricular	33 (27.3%)	**13(17.6%)**	**20 (42.6%)**	**<0.001**	
PVS (Doubal) (250)					
Basal ganglia					
1 (1-10)	109 (90.1%)	74 (100%)	35 (74.5%)	>0.05	
2 (11-20)	49 (40.5%)	18 (24.3%)	31 (66.0%)	>0.05	
3 (21-40)	15 (12.4%)	3 (4.1%)	11 (23.4%)	>0.05	
4 (>40)	14 (11.6%)	4 (5.4%)	10 (21.3%)	>0.05	
CSO					
1 (1-10)	55 (45.5%)	29 (39.2%)	26 (55.3%)	>0.05	
2 (11-20)	52 (43.0%)	29 (39.2%)	23 (48.9%)	>0.05	
3 (21-40)	37 (30.6%)	21 (28.4%)	16 (34.0%)	>0.05	
4 (>40)	17 (14.0%)	9 (12.2%)	8 (17.0%)	>0.05	
CMBs					
Lobar (1-4)	16 (13.2%)	**4 (7.8%)**	**12 (27.3%)**	**0.001**	**0.001**
Lobar (≥5)	9 (7.4%)	**1 (2.0%)**	**8 (18.2%)**	**0.001**	
Deep (1-4)	4 (3.3%)	**0**	**4 (9.1%)**	**<0.05**	0.05
Deep (≥5)	1 (0.8%)	0	1 (2.3%)	>0.05	
Cerebellar (1-4)	5 (4.1%)	2 (2.7%)	3 (6.4%)	>0.05	>0.05
Cerebellar (≥5)	0	0	0	>0.05	
CAA (according to Boston 2.0. criteria) (36)					
Possible	25 (20.7%)	14 (18.9%)	11 (23.4%)	>0.05	
Probable	10 (8.3%)	**0**	**10 (21.3%)**	**0.006**	
Charidimou's score**(189)	1 (0-1,5)	0 (0-1)	1 (1-2)	>0.05	
Other					
cSS (presence/absence) ***	7 (5.8%)	2 (2.7%)	5 (10.6%)	>0.05	
Atrophy					
GCA score	16.6 (± 5.9)	15.8 (±6.2)	17.6 (±5.4)	>0.05	
MTA (bilateral) score >1.5	63 (52.1%)	32 (43.2%)	31 (66%)	>0.05	

Data are displayed as the mean (±SD), median (IQR), or number of cases (%) as appropriate. Bold characters display statistically significant differences (p<0.05).
**One cortical microinfarct which was not considered sufficient to classify the patient as AD/VaD*
***Median and interquartile range*
**** All the cSS are focal (restricted to ≤3) except for one that corresponds to the AD/VaD group, which is disseminated (≥4 sulci).*

Other additional correlations initially reached statistical significance but did not withstand FDR correction. Notably, some of these trends aligned with clinical expectations. First, advancing age showed weak positive associations with PV and deep or SC WMHs, as well as with deep CMBs. Second, MTA exhibited weak and negative associations with MMSE scores, the cube copying test, and imitation of posture praxis. Third, and likely, GCA scores were weakly and negatively correlated with MMSE and total learning capacity. Fourth, severe PVS in the basal ganglia correlated weakly and negatively with total recognition performance. Fifth, PV WMHs exhibited a weak positive correlation with TMT A scores, reflecting worse cognitive performance.

Biofluid (plasma and CSF) BMs

The only statistically significant correlation that was found between plasma and CSF BMs was a weak positive correlation between plasma t-Tau and CSF p-Tau and t-Tau. However, this correlation did not remain statistically significant after FDR correction.

CSF BMs:

CSF biomarker levels did not differ significantly between men and women, either in the overall cohort or within each diagnostic group (AD and AD-CVD).
No statistically significant correlations were observed among CSF BMs and clinical, NPS, or imaging BMs across the whole cohort.

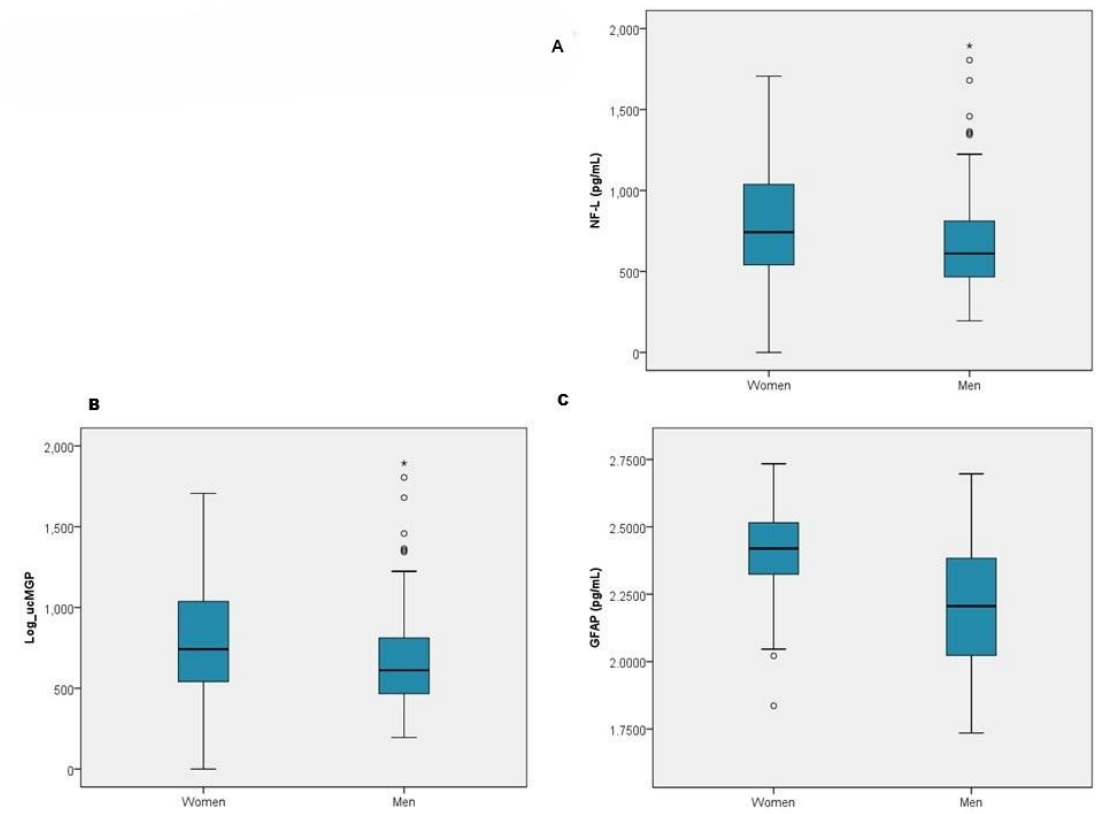
Blood BMs:

Effect of age and sex

Overall, blood BMs levels did not differ significantly between men and women, except for GFAP ($p<0.001$), NfL ($p=0.019$), and ucMGP ($p<0.001$), all of which were significantly higher in women than in men (Figure 13).

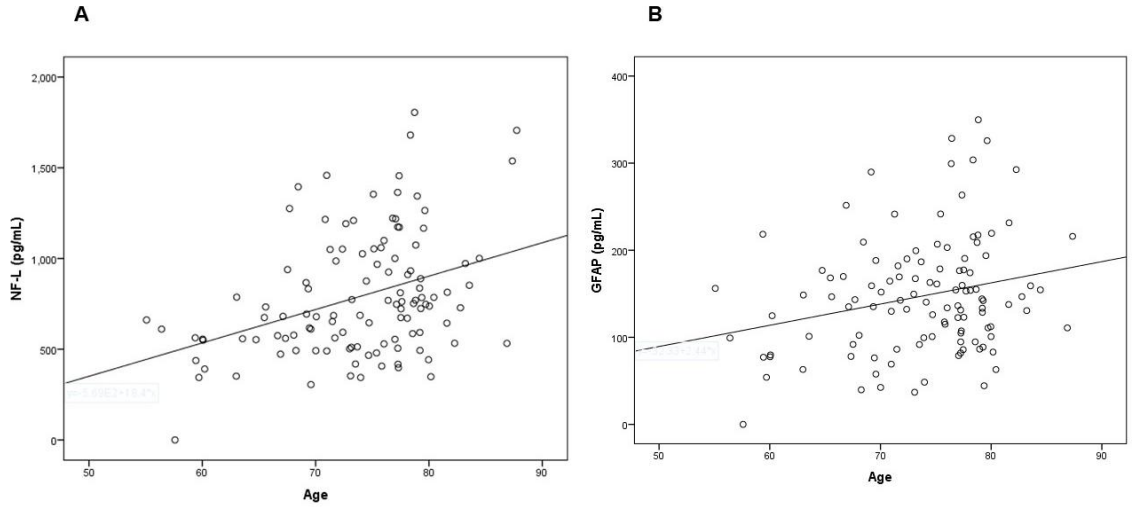
Higher levels observed in women were maintained for GFAP when comparing BM differences within each diagnostic group, specifically in the AD and AD-CVD groups ($p = 0.0068$ and $p=0.014$ correspondingly), and for ucMGP in the AD group ($p = 0.025$). No other significant sex-based differences in plasma BM levels were observed.

Figure 13. Comparison of NfL (A), ucGMP (B), and GFAP (C) by sex



As shown in Figure 14, NfL ($r=0.408$, $p<0.001$) and GFAP ($r=0.422$, $p<0.001$) values exhibited a moderate positive correlation with increasing age.

Figure 14. Correlations between NfL (A) and GFAP (B) and age



Other findings

As shown in table 13, ucMGP levels were lower in diabetic individuals (3.8±0.2 vs. 4±0.5, p=0.005). sTREM2 levels were lower in individuals with obstructive sleep apnoea (1.2±0.5 vs. 1.4±0.2, p=0.028) and in those with a history of stroke (1,4 (±0.2) vs 1.5 (±0.3), p=0.008).

Weak but positive associations were observed between sTREM2 and both lobar (r=0.35, p=0.02) and deep (r=0.3, p=0.04) CMBs, as well as between ucGMP and lobar CMBs (r=0.2, p=0.04). sTREM2 weakly and positively correlated with severe PVS in both the CSO and basal ganglia.

Table 13. sTREM2 and ucMGP in groups defined by comorbidities and risk factors

Variables		sTREM2		ucMGP	
Smoking status	Current	1.4 (±0.07)		3.8 (±0.1)	
	Previous	1.3 (±0.07)		3.9 (±0.05)	
	Never	1.4 (±0.03)		3.9 (±0.03)	
		Yes	**No**	**Yes**	**No**
Alcohol (usual consumption)		1.4 (±0.5)	1.4 (±0.3)	3.8 (±0.9)	3.9 (±0.3)
Hypertension		1.3 (±0.3)	1.4 (±0.5)	3.9 (±0.3)	4 (±0.5)
Dyslipidaemia		1.3 (±0.4)	1.4 (±0.3)	3.9 (±0.4)	4 (±0.4)
Diabetes mellitus		1.3 (±0.5)	1.4 (±0.3)	**3.8 (±0.5)**	**4 (±0.3)**
Ischaemic heart disease		1.5 (±0.6)	1.3 (±0.3)	3.9 (±0.7)	3.9 (±0.3)
Obstructive Sleep Apnoea		**1.2 (±0.5)**	**1.4 (±0.3)**	4 (±0.5)	3.9 (±0.3)
Previous stroke		**1.4 (±0.2)**	**1.5 (±0.3)**	3.8 (±0.7)	3.9 (±0.3)
ApoE carriership status		1.4 (±0.3)	1.4 (±0.4)	3.9 (±0.4)	3.9 (±0.4)

Bold characters display statistically significant differences (p<0.05).
Data are displayed as the mean (±SD).

Objective 2. AD vs AD-CVD

This subsection is concerned with the differences between the two diagnostic groups (AD and AD-CVD) across the diverse categories of data (demographic, clinical, NPS, imaging and biofluid BMs).

Demographic and clinical features and risk factors

As shown in table 10, there were only two statistically significant differences. First, the AD-CVD more commonly had a history of stroke (p=0.014). Second, the AD-CVD group had a higher share of homozygous individuals for E4 (17 vs 2%, p=0.01).

However, some non-statistically significant differences can be observed. There was a higher overall percentage of women in the AD group. The AD-CVD participants were older. There was a higher percentage of high blood pressure in the AD-CVD group, with all the other CVRF exhibiting very similar percentages in both groups. Results of the analysis after removing the individuals classified as AD-CVD solely for having extensive WMHs were similar.

As shown in Table 11, there were no significant differences between the two diagnostic groups in the primary clinical features of the disease, except for a higher conversion rate to dementia in the AD-CVD group compared to the AD group (40% vs. 23%, p=0.048). However, in the sensitivity analysis, this difference was no longer statistically significant (32% vs 23%, p=0.3).

Additionally, a trend suggesting a higher prevalence of non-memory symptoms -particularly dysexecutive symptoms (19.1% vs. 5.4%; p= 0.056)- as the initial manifestation in the AD-CVD group was observed.

NPS

No significant differences were found in NPS test performance between the AD and AD-CVD groups, with the exception to a trend (p=0.068) in semantic fluency to be lower in the AD-CVD group. Similar results were observed after excluding participants classified as AD-CVD solely due to extensive WMHs (semantic fluency test: p = 0.059).

Imaging BMs

As expected, and as summarised in Table 12, the AD/CVD had a higher percentage of CVD BMs, such as total (p=0.04), deep (p=0.02), and territorial (p=0.04) ischaemic infarcts, as well as lobar (p=0.001) CMBs, and WMH of any type and severity. However, there were two parameters -severe PVS in the two locations assessed (basal ganglia

and CSO) and atrophy measures -that were similar between the two groups. No additional statistically significant differences were identified after excluding participants classified as AD-CVD based solely on extensive WMHs.

Biofluid BMs:

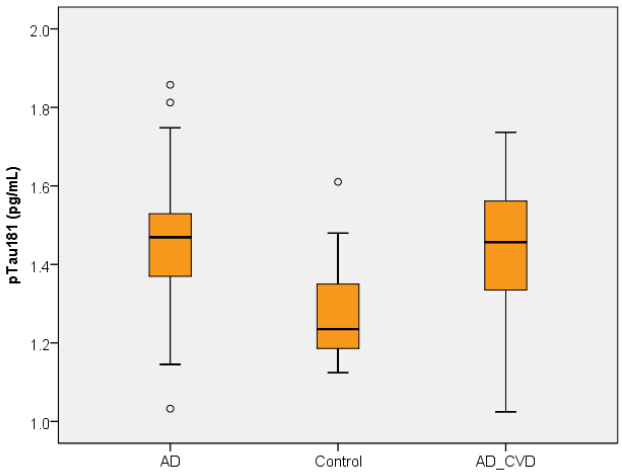
CSF BMs:

No statistically significant differences in CSF BM levels were found between the two diagnostic groups (AD and AD-CVD), even when participants classified as AD-CVD solely based on extensive WMHs were removed.

Blood BMs:

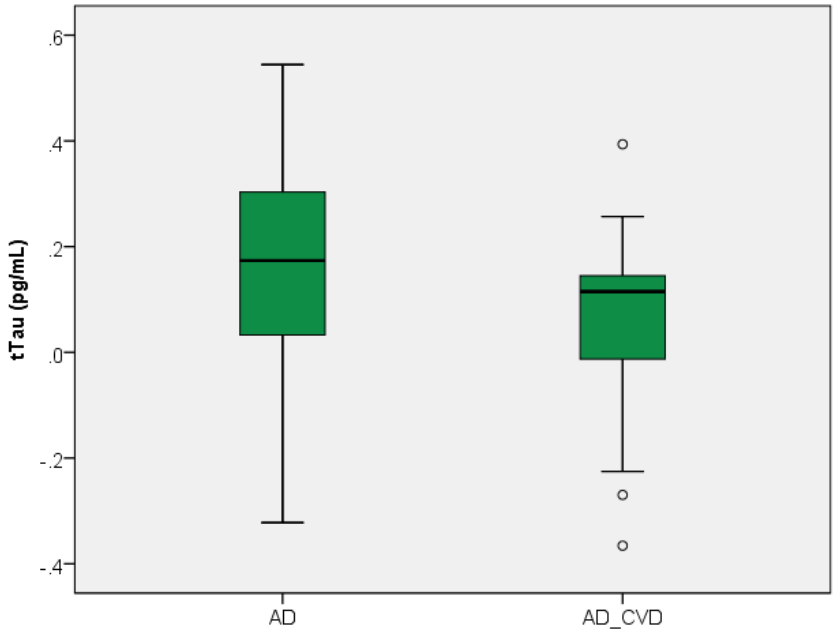
In contrast to the control group, both the AD and AD-CVD groups exhibited lower Aβ-40 levels (p=0.002 for both). Moreover, the AD-CVD and AD groups had higher p-tau 181 (p<0.001 for AD, p=0.007 for AD-CVD) (Figure 2), GFAP (p<0.001 for both) and NfL (p=0.002 for AD, p<0.001 for AD-CVD) levels than the control group. Total tau (t-Tau) was higher in the AD-CVD group compared to controls (p=0.034). No statistically significant differences in sTREM2 levels were observed.

Figure 15. p-tau181 levels (mean+/-2 SD) in the control, AD and AD-CVD groups



No statistically significant differences were observed between the AD and the AD-CVD groups. However, plasma t-Tau levels approached statistical significance (p=0.056), with the AD group exhibiting higher levels (Figure 16). This difference became statistically significant (p=0.02) after excluding participants classified as AD-CVD based solely on extensive WMHs (p=0.02).

Figure 16. t-tau levels (mean+/-2 SD) in AD and AD-CVD



Objective 3. Diagnostic value of BMs

This subsection presents the results related to the third objective: evaluating the diagnostic performance of the different BMs investigated.

NPS tests

No single NPS test effectively predicted whether a participant had AD or AD-CVD, as all AUC values from the ROC curves constructed using NPS tests remained below 0.7. The inclusion of ApoE4 carriership status in the model did not modify the ROC curves.

Biofluid BMS

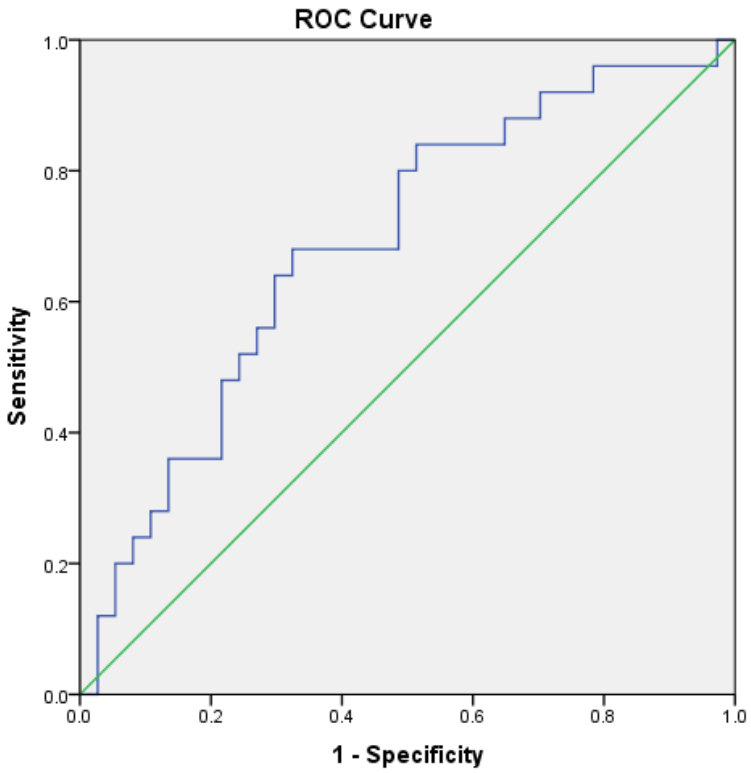
CSF BMs:

None of the CSF BMs demonstrated strong predictive capacity in distinguishing AD from AD-CVD; that is, none of the cutoff points assessed in the logistic regression models achieved an AUC of 0.7 or higher in distinguishing AD from AD-CVD. These findings remained consistent after excluding participants classified as AD-CVD based solely on extensive WMHs.

Blood BMs:

The diagnostic performance of ucGMP for distinguishing AD from AD-CVD yielded an AUC of 0.706 (Figure 17). All other blood BMs had AUC values below 0.7.

Figure 17. ROC curve for plasma ucGMP to distinguish AD from AD-CVD



However, when participants classified as AD-CVD based solely on extensive WMHs were excluded from the analysis, AUC values exceeding 0.7 were observed for Aβ-42 (0.7), Aβ-40 (0.7), t-Tau (0.74), sTREM2 (0.71), and ucMGP (0.72).

Objective 4. Prognostic value of the BMs

This subsection is concerned with the fourth objective: exploring the performance of the different BMs used in this study to predict clinical prognosis of the participants.

Baseline MMSE scores showed a positive correlation- with a low to moderate strength- with total learning capacity (r=0.439, p=0.004), and with phonetic (r=0.413, p=0.006) and semantic fluency (r=0.379, p=0.01). MMSE scores at the follow-up only showed a weak positive correlation with retention index (0.42, p=0.03) and phonetic fluency (r=0.379, p=0.03).

GDS score at baseline showed a weak negative correlation with total learning capacity (r=-0.385, p=0.033). GDS at follow-up showed weak negative correlations with total learning capacity (-0.303, p=0.006), total recognition (r=-0.391, p=0.006) and cube tests score (r= -0.384, p=0.049). In contrast, GDS score at baseline did not show significant correlations with NPS tests after correction by FDR.

As outlined in the cohort description, some correlations were found among worse cognitive performance and greater medial temporal and general atrophy scores, as well as with a higher burden of PVS in the basal ganglia and of PV WMHs. Additionally, advancing age weakly and negatively correlated with baseline MMSE scoring. However, none of these associations remained statistically significant after FDR correction.

Objective 5. CAA

Finally, this subsection summarises the results concerning the fifth objective of the study: describing the frequence of CAA in our sample and its relationship with the BMs used in our study.

Among the 95 patients who underwent an MRI, 35 met the criteria for either possible or probable CAA, with 10 fulfilling the criteria for the probable category. As outlined in the methods section, all cases of probable CAA were classified as AD-CVD.

Charidimou's score was not significantly correlated to any clinical, NPS or biofluid BM.

RESULTS STUDY TWO

The results of the study were published in the scientific journal European Journal of Neurology. A capture of the first page of the article, which is freely accessible at: https://onlinelibrary.wiley.com/doi/full/10.1111/ene.16404, is provided in Appendix 1.

As shown in the study flowchart (figure 18), a total of 976 individuals participated in the baseline visit. The acceptance rate at baseline was 72.4%.

Of the 483 participants who were invited for the follow-up MRI, the MRI was completed in 345 individuals. Thus, the final sample size at follow-up included 345 participants with valid imaging and cognitive data from both time points.

Figure 18. Flowchart Study Two

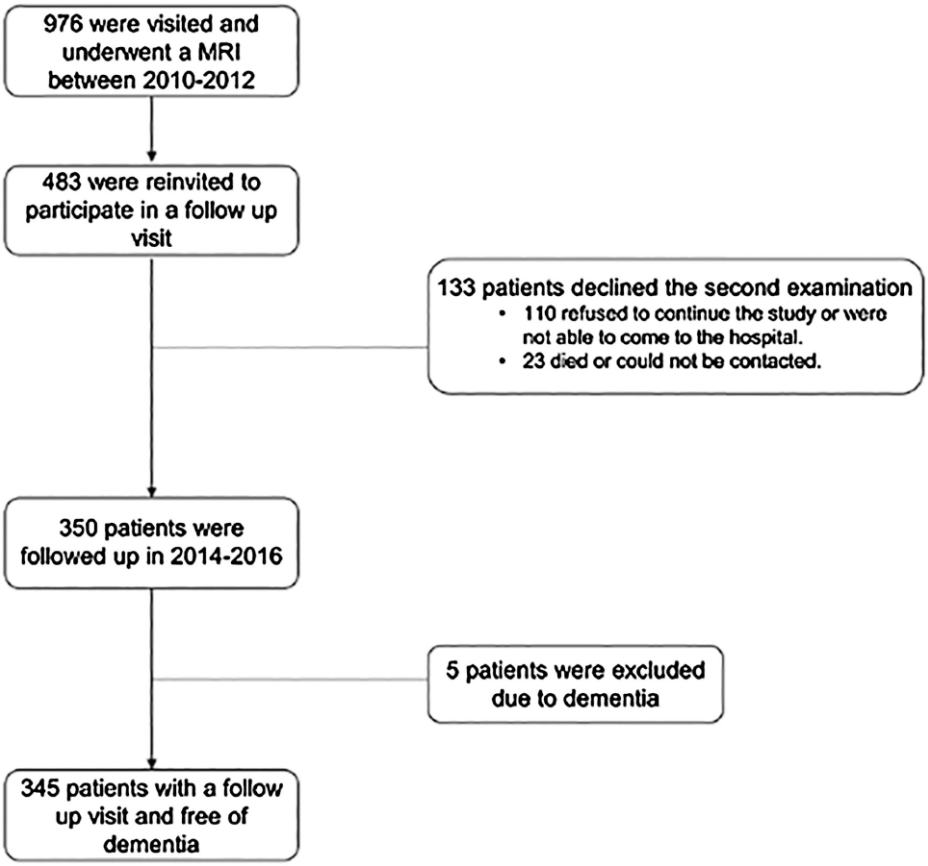


Table 14 shows the median levels of the pollutants analysed.

Table 14. APs levels and their established limits*

NO2 (ug/m3)	57.1 (53.4-59.9) †	10‡	40§
NOx (ug/m3)	92.5 (92.2-95.9) †		
$PM_{2.5}$ (fine) (ug/m3)	16.0 (14.4-17.0) †	5‡	25§
PM_{10} (ug/m3)	33.7 (31.4-35.9) †	15‡	40§
$PM_{2.5\ abs}$ (10^{-5}/m3)	2.77 (2.74-2.96) †		
PM_{coarse} (ug/m3)	1.0 (17.6-20.2) †		

**All values (including World Health Organization and European Union guidelines) are for long-term exposure (annual).*

†Median and interquartile range (IQR) of the values estimated for all the cohort at baseline (n = 966).

‡ Limits established by the World Health Organization in Global Air Quality Guidelines 2021.

§ Limits established by the European Union in ambient air quality and clean air for Europe standards in 2008.

Significant correlations between all APs were found (all of them $p<0.001$). Correlation coefficients between gases (NO2 and NO_x) and particles ranged from 0.14 to 0.6, whereas correlations among particles were stronger (between 0.58 and 0.75).

General characteristics of the cohort

The median age of participants was 64 (54, 67) years. A total of 49.4% were men. Participants had a median of 8 (7,12) years of education.

Median systolic blood pressure was 142 (132 to 155) mmHg and median diastolic blood pressure (DBP) was 78 (71 to 85) mmHg, indicating that participants were reasonably well-controlled despite being hypertensive. Patients had had an arterial hypertension diagnosis for a mean of 10.5 (SD 7.8) years, and many (55%) were treated with 2 or more BP lowering drugs. Abdominal obesity was the most prevalent vascular risk factor

(69%) following hypertension. The mean low-density lipoprotein level was 128.9 (±34.0). 225 had dyslipidaemia (28.2%), 229 (23.5%) were diabetic and 148 (15.2%) were active smokers. A total of 38 patients (3.9%) had an atrial fibrillation.

As summarized in Table 15, people with dyslipidaemia and AF were exposed to higher median levels of $PM_{2.5}$. A weak positive correlation between DBP and PMcoarse levels was found, as well as a weak negative correlation between DBP and NO2, NO_x and $PM_{2.5}$ absorbance. We did not find any other associations between exposure to AP and vascular risk factors at baseline, nor with established cardiovascular or renal disease or AF.

Table 15. Distribution of AP concentrations by vascular risk factors at baseline

Vascular Risk Factor	NO2(ug/m3)	NO_X (ug/m3)	$PM_{2.5}$ (ug/m3)	$PM_{2.5abs}$ (10^{-5}/m3)	PM_{coarse}(ug/m3)	PM_{10} (ug/m3)
Dyslipidaemia						
Yes (275)	57.7 (54.4, 60.3)	92.8 (92.3, 100.0)	**16.3 (14.9, 17.3) ***	2.8 (2.75, 3.1)	19.3 (17.7, 20.3)	34.0 (31.6, 36.4)
No (696)	56.7 (54.5, 60.0)	92.6 (92.3, 94.8)	**15.4 (14.2, 16.7) ***	2.8 (2.7, 3.1)	18.8 (17.7, 20.1)	33.5 (31.2 vs 35.9)
Diabetes						
Yes (229)	57.2 (54.4, 60.3)	92.5 (92.2, 95.6)	16.0 (14.8, 17.9)	2.8 (2.7, 3.0)	19.2 (17.5, 20.3)	33.8 (31.4, 36.3)
No (976)	57.5 (54.4, 60.3)	92.8 (92.2, 98.3)	16.1 (14.6, 17.0)	2.8 (2.7, 3.1)	19.2 (17.8, 20.3)	33.9 (31.5, 36.1)
Abdominal obesity						
Yes (698)	57.4 (54.0, 60.2)	92.7 (92.2, 97.7)	16.1 (14.6, 17.2)	2.8 (2.7, 3.0)	19.2 (17.8, 20.4)	34.0 (31.5, 36.7)
No (262)	57.3 (54.4, 59.8)	92.7 (92.3, 96.1)	16.2 (14.6, 17.0	2.8 (2.7, 3.1)	19.3 (17.7, 20.1)	33.7 (31.2, 35.9)
Current smoker						
Yes (148)	57.9 (54.4, 61.7)	93.1 (92.3, 97.6)	16.4 (14.8, 17.7)	2.8 (2.7, 3.0)	19.3 (17.7, 20.4)	33.8 (32.1, 36.5)
No (827)	57.3 (54.4, 60.0)	92.7 (92.2, 97.6)	16.1 (14.6 ,17.0)	2.8 (2.7, 3.0)	19.2 (17.7, 20.4)	33.9 (31.4, 36.1)
REGICOR						
High (≥10) (665)	57.4 (54.2, 59.4)	92.7 (92.5, 96.0)	16.2 (14.9, 17.0)	2.8 (2.7, 3.0)	19.2 (17.8, 20.5)	33.8 (31.6, 36.3)
Low (0-9) (297)	57.3 (54.4, 60.5)	92.7 (92.2, 99,2)	16.0 (14.5, 17.1)	2.8 (2.7, 3.0)	19.2 (17.6, 20.2)	33.9 (31.3, 36.0)
Atrial Fibrillation						
Yes (38)	57.5 (55.5, 64.7)	93.3 (92.3, 98.0)	**16.8 (15.1, 18.8) ***	2.8 (2.7, 3.4)	19.5 (17.5, 20.9)	33.9 (31.3, 36.0)
No (938)	57.3 (54.8, 60.0)	92.7 (92.3, 97.6)	**16.0 (14.6, 17.0) ***	2.7 (2.7, 3.0)	19.2 (17.8, 20.3)	33.9 (31.4, 36.0)

Values showed are the medians and the interquartile ranges obtained.

***:** indicates for p-value<0.05

AP and prevalence of covert cerebrovascular disease

At the baseline visit, 99 patients (10.1%) had CBI, 65 (6.7%) had CMB, 91 (9.3%) had extensive PV WMHs, and 47 (4.8%) had extensive subcortical or deep WMH.

As shown in Table 16 exposure to $PM_{2.5}$ was related to higher odds of having CBI (OR 2.21; CI95% 1.06-4.60, p=0.03, n=976)), and NO2 of having extensive WMH both in the subcortical (OR 1,66; CI95% 1,17-2,35, p<0.01) and periventricular (OR 1,96; CI95% 1,10-3,50, p=0.02, n=976) areas. $PM_{2.5\ absorbance}$ was associated with higher odds (OR 1,72 (1,13-2,60), p=0,01, n=976) of having extensive SC WMH. No associations were found between any of the pollutants and the presence of CMBs or extensive EPVS at basal ganglia or CSO at baseline.

Table 16. Association between ambient air pollutants and brain imaging results at baseline

	CBIs (99/889)		**Extensive†SC WMHs** (47/882)		**Extensive† PV WMHs** (15/882)	
	OR	**P value**	**OR**	**P value**	**OR**	**P value**
NO2	1.13 (0.90-1.40)	0.28	**1.66 (1.17-2.35)**	**<0.01**	**1.96 (1.10-3.50)**	**0.02**
NOx	1.04 (0.92-1.17)	0.56	1.17 (0.99-1.38)	0.05	1.24 (0.96- 1.60)	0.09
$PM_{2.5}$	**2.21 (1.06-4.60)**	**0.03**	1.59 (0.56-4.45)	0.38	1.98 (0.34-11.6)	0.45
$PM_{2.5\ abs}$	1.16 (0.82-1.64)	0.39	**1.72 (1.13-2.60)**	**0.01**	1.01 (0.70-1.45)	0.27
PM_{10}	1.35 (0.74-2.46)	0.32	2.11 (0.99-4.49)	0.05	2.41 (0.69-8.39)	0.17
PM_{coarse}	2.48 (0.80-7.67)	0.11	1.43 (0.31-6.58)	0.64	1.52 (0.09-25.2)	0.77

CBI= Covert Brain Infarcts; PV WMH: Periventricular White Matter Hyperintensities; SC WMH: Subcortical White Matter Hyperintensities.

Values showed are the Odds Ratio obtained in the multivariate models, followed by p-value. Covariates included age, sex, years of education and REGICOR score. Missings in covariates were as follow: Air pollutants (n=10), REGICOR score (n=15), PV VWHs (n=18), SC WMHs (n=18), years of education (n=65).

† Extensive SC WMHs included 2 and 3 Fazekas score, whereas extensive PV WMHs included score 3.

In multipollutant models, increases in 10 ug/m3 $PM_{2.5}$ exposure doubled the risk of CBIs independently of VRF, education and all other pollutants, whereas extensive WMHs (both in PV and SC areas) were only related to NO2 exposure.

AP and incidence of covert cerebrovascular disease (changes or progression)

The most frequent change in cerebrovascular lesions was marked subcortical WMH progression, which was observed in 71 patients (19.7%). The incidence of other lesions (incident CBI, incident CMB and marked WMH PVH progression) was 5-6% of the sample each.

As shown in Table 17, NO2. was related with higher odds of developing marked subcortical WHM progression (OR 1,4; CI95% 1,05-19; p=0,02, n=317) and so did NO_x with incident CMBs (OR 1,36; CI95% 1.04-1.79; p=0,02, n=317).

When the combination of new lesions was considered as a burden score, we found a radiological progression in about 35% of the patients at follow-up (63.9% no progression; 26.2% one lesion and 9.9% two or more lesions). With this approach, NO2 (OR 1,33; CI95% 1.04-1.68; p=0.019, n=317) and NO_x (OR 1,18; CI95% 1.03-1.35; p=0.015, n=317) were the only APs that related to higher odds of progression. More specifically, we found that each increase in 10 units of NO2 or NO_x was equivalent to ten years of aging in this cohort.

Table 17. Association between ambient air pollutants and brain imaging results at follow up

Pollutant	Odds ratio	95% Confidence interval	P value
NO2			
Incident infarcts	1.38	0.79-2.39	0.38
Marked† PV WHS progression	1.04	0.62-1.73	0.87
Marked† SC WHS progression	**1.4**	**1.05-1.9**	**0.02**
Incident CMBs	1.43	0.78-2.63	0.24
Progression total score	**1.32**	**1.0-1.67**	**0.02**
NO_X			
Incident infarcts	1.25	0.96-1.63	0.10
Marked† PV WHS progression	0.95	0.73-1.23	0.68
Marked† SC WHS progression	**1.13**	0.97-1.31	0.24
Incident CMBs	**1.36**	**1.04-1.79**	**0.02**
Progression total score	**1.18**	**1.03-1.34**	**0.01**
$PM_{2.5}$			
Incident infarcts	3.11	0.79-12.28	0.11
Marked† PV WHS progression	0.45	0.06-3.02	0.41
Marked† SC WHS progression	0.92	0.37-2.30	0.86
Incident CMBs	1.01	0.19-5.44	0.99
Progression total score	1.00	0.46-2.15	0.99
$PM_{2.5\ abs}$			
Incident infarcts	1.16	0.53-2.56	0.71
Marked† PV WHS progression	0.96	0.40-2.31	0.93
Marked† SC WHS progression	1.4	0.93-2.12	0.11
Incident CMBs	1.30	0.57-2.97	0.54
Progression total score	1.28	0.89-1.82	0.17
PM_{10}			
Incident infarcts	1.91	0.24-3.55	0.89
Marked† PV WHS progression	0.36	0.07-1.92	0.23
Marked† SC WHS progression	0.95	0.45-1.98	0.89
Incident CMBs	1.01	0.23-4.32	0.98
Progression total score	0.83	0.44-1.54	0.56
PM_{coarse}			
Incident infarcts	1.23	0.12-12.68	0.86
Marked† PV WHS progression	0.17	0.01-2.26	0.18
Marked† SC WHS progression	0.48	0.13-1.79	0.27
Incident CMBs	1.21	0.09-15.80	0.89
Progression total score	0.49	0.16-1.47	0.20

Values showed are the Odds Ratio obtained in the multivariate models, followed by their p-value. Covariates were age, sex, years of education, and REGICOR score. Valid sample size was 346 for incident infarcts, 342 for PV or SC marked WMH progression, 343 for BMBs and 342 for the progression score.

AP and cognition

We did not find any association between AP and cognitive function (global, memory or executive) or status (MCI versus NA) at baseline or follow-up (Table 18 and Table 19). These findings remained consistent after adjusting for baseline cognitive performance

and the presence of CVD. Specifically, we adjusted for WMH progression over the follow-up period, as it has been identified as a determinant of cognitive transitions in previous research (15).

Table 18. Association between exposures to APs and cognition at baseline

BASELINE	Global function	Executive function	Memory
NO2	0,003 (-0,002 a +0,008)	0,002 (-0,002 A 0,006)	-0,001 (-0,005 A 0,004)
Nox	0,001 (-0,002 a 0,004)	0,002 (-0,001 a 0,004)	0,000 (-0,003 a 0,002)
PM2.5	0,005 (-0,012 a 0,023)	0,010 (-0,005 a 0,025)	0,011 (-0,004 a 0,026)
PM2.5 abs	0,032 (-0,053 a 0,116)	0,031 (-0,039 a 0,102)	0,003 (-0,069 a 0,074)
PM10	0,020 (-0,047 a 0,006)	-0,007 (-0,030 a 0,015)	-0,001 (-0,014 a 0,012)
PMcoarse	-0,009 (-0,024 a 0,006)	-0,006 (-0,019 a 0,007)	-0,001 (-0,023 a 0,021)

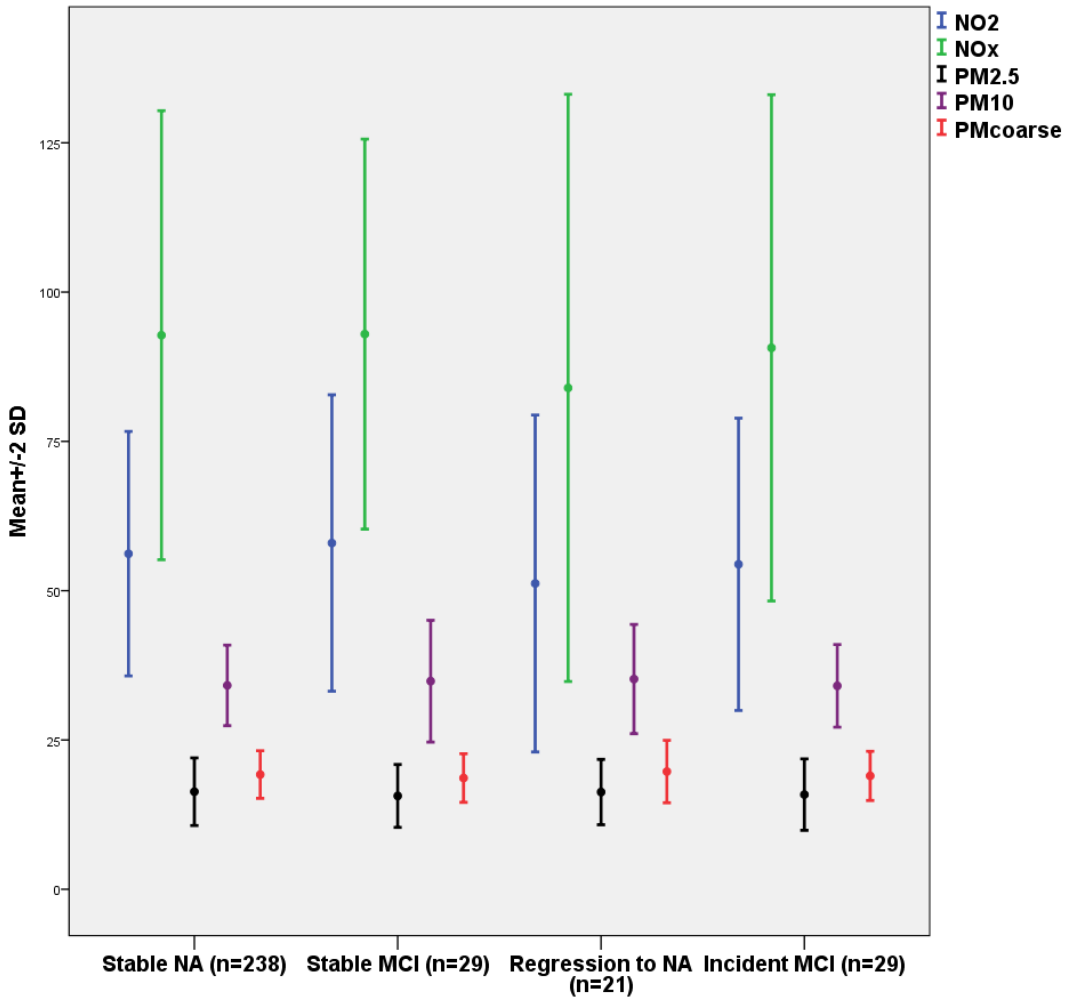
Table 19. Association between exposures to air pollutants and cognition at follow-up

FOLLOW-UP	Global function	Executive function	Memory
NO2	0,002 (-0,005 a 0,009)	0,001 (-0,006 A 0,007)	0,001 (-0,008 a 0,010)
Nox	0,001 (-0,003 a 0,004)	0,000 (-0,003 A 0,004)	0,001 (-0,004 a 0,005)
PM2.5	0,010 (-0,015 a 0,034)	0,012 (-0,011 A 0,035)	0,006 (-0,025 a 0,038)
PM2.5 abs	-0,033 (-0,146 a 0,081)	-0,067 (-0,175 A 0,040)	0,019 (0,129 a 0,167)
PM10	0,005(-0,016 a 0,026	0,001 (-0,018 A 0,021)	0,005 (-0,022 a 0,032)
PMcoarse	0,025 (-0,011 a 0,060)	0,024 (-0,009 A 0,057)	-0,006 (-0,051 a 0,040)

Global cognitive function at baseline for the full cohort had a median DRS-2 total score of 11 (interquartile range: 9–12). At baseline, 74 participants (7.6% of the sample) were diagnosed with MCI.

Over time, most of the individuals in the sample remained in the same cognitive status group. Concretely, 238 individuals were classified as stable NA and 29 as stable MCI. However, several transitions in cognitive status were also noted: 29 as incident MCI (NA to MCI) and 21 as regressors to NA (MCI to NA). Nevertheless, as shown in figure 19, none of the APs were associated with cognitive transitions over the follow-up, with considerable overlap of AP levels between all groups.

Figure 19. Cognitive transitions during follow-up



The figure illustrates the exposure to the five air pollutants (APs) analysed expressed in ug/m^3 in the four cognitive transitions groups: stable normal ageing (NA) (n=328), stable mild cognitive impairment (MCI) (n=29), regression to normal (MCI at baseline and NA at follow-up) (n=21) and incident MCI (NA at baseline and MCI at follow-up) (n=29). PM 2.5 abs is not included because it is expressed in 10^{-5}/m3.

DISCUSSION

The discussion will be organised in two parts.

The first part will be focused on the first overarching aim, concerned with strengthening the evidence base regarding the characteristics and progression of AD-CVD.

The second part will be focused on the second overarching aim, which is to study the role of AP -a common risk factor for both cognitive impairment and CVD- in cognition and cCVD.

First part of the discussion (Study One)

In study One, we have used clinical and paraclinical data (BMs) to describe individuals with AD-CVD and compare them with individuals with pure AD.

In summary, the only significant difference between groups was a faster functional decline in the AD-CVD group, although this was not observed in the sensitivity analysis. In this analysis, plasma t-tau levels were higher in the pure AD group. Across the entire cohort, extensive WMHs were correlated with more severe medial temporal atrophy, and approximately one third of participants fulfilled criteria for either possible or probable CAA.

With respect to biofluid BMs, the only one that showed an acceptable (AUC>0.7) discriminative capacity in distinguishing AD from AD-CVD was ucMGP. However, in the sensitivity analysis, t-tau (0.74), sTREM2 (0.71), and ucMGP (0.72) also achieved AUC greater than 0.7. We also found several interesting associations between biofluid and imaging BMS. sTREM2 was associated with non-specific CVD BMs, and ucGMP with lobar CMBs.

This study was inherently complex for several factors. First, both “CVD” and “VCI” encompass broad spectrums of diseases and underlying, often inter-related, pathophysiological processes (45). Second, co-pathologies are, while common, a still relatively poorly understood and relatively overlooked aspect of cognitive impairment. Elucidating the role each of the pathologies is having in the clinical picture of the patient is fraught with difficulty.

While BMs offer a valuable opportunity to more accurately address these challenges, they also have several limitations -including a relative neglect to date of non-AD pathologies-, as well as several considerations that should be taken into account when they are used. Likely, current diagnostic frameworks are mostly focused on AD and may be insufficient to fully and accurately comprehend mixed conditions such as AD-CVD.

The main results of Study One -organized around its five objectives- will be addressed, and their relevance and implications for future research directions will be discussed. Later, the discussion will draw on the complexities and limitations of Study One to put further in context its results and the interpretation we have made of them.

Description of the cohort

Clinical and demographic

The clinical demographic characteristics of the full cohort were consistent with our eligibility criteria: MCI or mild dementia due to AD, whether associated or not with significant CVD. The burden of traditional CVRF is consistent with the demographic features of the sample, which in turn are consistent with AD epidemiology (183).

The existing heterogeneity in the final diagnosis of the patients who were excluded of the study for not meeting the enrolment criteria (having positive CSF AD BMs) highlights the often-unspecific nature of broad clinical categorisations such as “MCI”.

The clinical picture of the participants was largely consistent with that of AD, including the observed decline in the MMSE over a median follow-up of 12 months in both groups. Nonetheless, we acknowledge that the clinical manifestations of VCI are often more heterogeneous and non-specific, which may complicate the identification of distinct patterns.

Imaging BMs

Interestingly, 16 patients classified as AD had brain infarcts in neuroimaging consistent with cCVD, but these did not fulfil the criteria we applied- which are a combination of criteria currently in use- for classifying them as AD-CVD. Further, the prevalence of WMHs Fazekas 1 and PVS both in the CSO and basal ganglia is high in both the AD and the AD-CVD groups. Moreover, 5 patients classified as AD also had strictly lobar CMBs, 2 had focal cSS, and 11 met Boston 2.0. criteria for the category of “possible CAA”.

ApoE4

We have sought to determine ApoE status in our study acknowledging that, beyond its role for risk stratification, ApoE may have diagnostic utility (as part of multimodal BMs models (231)), and prognostic (further addressed below) and therapeutic implications (e.g., due to the higher risk of ARIAs).

The prevalence of ApoEε4 carriership and ApoEε4 homozygosity in our study was consistent with that reported for individuals with high or intermediate AD neuropathological changes in the National Alzheimer’s Coordinating Centre (NACC) database (53.7% vs 52.1% and 8.7% vs 11.6%, respectively) (139,140). The former was particularly high in those with a family history of dementia (71%) and in those classified as AD-CVD solely for having extensive WMHs (71.4%). This aligns with prior research, in which ApoE ε4 allele has been associated with a higher parietal WMH load in individuals with AD (185). Therefore, the AD phenotype with extensive WMHs and

without any other significant cerebrovascular lesion may have been at least partly driven by genetic factors -and, specifically, to the ApoE ε4 allele- which has been linked, in addition to AD, to microvascular disease, meningeal lymphatic and glymphatic dysfunction, and/or CAA (117,123,186,187), as stated above.

Biofluid BMs

Significant correlations between CSF and blood BMs were not observed, which contrasts with prior findings (188).

Increasing age was positively correlated with both GFAP and NfL levels, as previously described (189). This is consistent with the increasing prevalence of neurodegenerative disorders and CVD pathology in older populations.

Regarding sex differences, in our study we found that blood levels of GFAP, NfL, and ucGMP were higher in women than in men. Elevated GFAP levels in women have been reported in both AD and TBI cohorts (190). In contrast, previously reports regarding sex differences in NfL levels are conflicting, since higher levels have been reported in women with PD but, conversely, lower levels have been reported in women with AD (190,191)). To the best of our knowledge, sex-related differences in ucGMP have not been previously reported. Therefore, further research should specifically address sex-related differences regarding ucGMP and NfL, as well as the potential concomitant factors influencing the levels of these BMs.

In our study, lower ucMGP levels were found in diabetic individuals. This is consistent with previous research, in which lower levels have been associated with cardiovascular mortality and events (100).

Differences between AD and AD-CVD

We observed a higher rate of having a history of stroke, as expected, as well as a higher conversion to dementia in the AD-CVD group. The latter finding will be discussed in the prognosis subsection of the discussion. There was also a higher rate of ApoEε4 homozygous individuals in the AD-CVD group, likely partly related to the classification of individuals with extensive WMHs as AD-CVD.

With regards to biofluid BMs, no significant differences were detected between the AD and the AD-CVD groups, except for a trend toward higher blood t-tau levels in the AD group. This difference became statistically significant in the sensitivity analysis. This finding may indicate a lower degree of neurodegeneration in the AD-CVD group and could align with the additive hypothesis, which, as introduced before, posits that a lesser degree of AD neuropathology may be sufficient to produce clinical symptoms in the presence of significant CVD. However, caution is needed in its interpretation, which must be done within an appropriate clinical context, as the accuracy of plasma t-tau for detecting CNS neurodegeneration is lower than its CSF counterpart, given that its levels may be influenced by peripheral sources (80), as mentioned above.

Since these were the only differences we observed between the two groups, it could be argued that we cannot accept our hypothesis – that there would be features that would allow to accurately distinguish both conditions.

This lack of any other significant differences aligns with findings reported by Skillbäck et al (89). In their analysis of CSF AD biomarkers in 5,676 individuals across nine diagnostic categories -including early- and late-onset AD, FTD, LBD, VaD, and AD-CVD- they found comparable levels of CSF AD BMs in the AD and AD-CVD groups. A key finding from their study was that a substantial proportion of individuals in non-AD diagnostic categories exhibited biological evidence of AD pathology. Similar results were reported by Eckerström et al. (103), who analysed biofluid BMs in 493 participants with MCI.

Participants were classified into four groups: AD (based on NINCDS-ADRDA criteria (192); svCVD-related cognitive impairment (according to Erkinjuntti criteria(193)); AD-CVD- defined as participants meeting AD criteria in addition to a Fazekas score of 2 or 3 and/or a predominant dysexecutive presentation; and a control MCI group. Their results showed that the AD-CVD group exhibited a CSF biomarker profile similar to that of the AD group.

Assuming that we have not committed a Type II error, the similarity between the two groups -along with the widespread presence of at least some degree of co-pathology across the entire sample outlined above- may reflect the possibility that AD-CVD is better conceptualised as a continuum rather than as a clearly distinguishable, dichotomic category, as previously argued (60).

BMs with diagnostic capacity to differentiate AD from AD-CVD

Even if AD-CVD is conceptualized as a continuum rather than as a discrete pathological entity, distinguishing between more “AD-like” and more “VCI-like” profiles within the AD-CVD continuum can have clinical utility and diagnostic, prognostic, and therapeutic implications. For instance, this stratification can serve as an indicator of the extent to which AD pathology is contributing to the patient’s cognitive impairment- information that could be valuable, for example, in evaluating the risk-benefit profile of initiating DMTs for AD.

In addition to ucGMP, both sTREM2 and t-Tau showed acceptable discriminative performance in the sensitivity analysis. Although Aβ-40 and Aβ-42 individually reached an AUC of 0.7 as well in such analysis, they are more reliable when expressed as ratios, which helps account for interindividual variability (e.g., differences between high and low peptide producers).

These observations are consistent with ucGMP serving as a BM of vascular calcification, as well as with the observed differences in t-tau levels between AD and AD-CVD groups in the sensitivity analysis. T-tau (reflecting neurodegeneration) and ucGMP (associated with lobar CMBs in our study)- may be valuable tools to support the differentiation between more “AD-like” and more “VCI-like” profiles within the AD-CVD continuum, including CAA cases, and thus warrant further investigation.

With regard to sTREM2, Tsai et al. (94) reported that blood sTREM2 levels were positively correlated with WMHs, irrespective of amyloid and tau, and were also positively associated with PET-tau. Both findings align with our results.

First, concerning association with WMHs, the associations we found with CMBs and PVS are also consistent with sTREM2 being a non-specific BM of svCVD. However, it perhaps should be combined with other BMs, such as PET tau, for more appropriate interpretations. Further, these findings contrast with a study which did not find differences in CSF sTREM2 levels between AD, controls and CAA (42).

Second, considering association with PET-tau, the fact that sTREM2 demonstrated acceptable discriminative capacity in our sensitivity analysis may support the additive hypothesis, since individuals with "pure AD" would be expected to exhibit a greater burden of cortical tau -reflecting a more advanced stage of the AD pathological process- whereas those classified as AD-CVD would be expected to show a similar clinical profile with an earlier stage of AD pathology.

However, the changing pattern of sTREM2 along the evolution of AD (92) may difficult the interpretation of the clinical significance of this BM.

In our study, different combinations of BMs and/or clinical data did not significantly improve their discriminative performance. However, it has been argued that the development of new BMs and models based on multiple variables, along with the integration of BMs from different modalities and types -including imaging techniques and biofluids- holds significant promise for improving their interpretation and addressing existing limitations (194,195). Several such combinations have been explored, particularly those incorporating blood-based BMs, including a combination of pTau217, age and ApoE4 status (196), the "Amyloid Probability Score" (APS), consisting on age, ApoE status and blood AB42/40 measured by mass spectrometry (184) or the APS2, consisting in the ratio of pTau217 to non-p-tau217 combined with the AB42:AB40 ratio in plasma, analysed as well by mass spectrometry (197). Both APS and APS2 have demonstrated clinical utility and good performance in clinical settings (197,198).

Since the diagnostic utility of the BMs used, either alone or in combination, was modest, future studies will have to determine whether specific clinical, NPS, or biofluid BMs can reliably and validly differentiate between AD-CVD and "pure" AD cases.

BMs with prognostic capacity

We acknowledge that prognostic information in diseases impairing cognition should always be interpreted and communicated with caution, given the wide range of factors influencing their outcomes (199). Nevertheless, we believe that some interesting findings in this regard, described below, were observed in our study.

As expected, scores from the **GDS and MMSE** both at baseline and follow-up were correlated with poorer performance in formal NPS assessment (168,169).

Both scales were correlated as well with temporal and general atrophy scores. Although these associations did not withstand correction for FDR, they are consistent with the findings described by Wan et al (200), who concluded that the MTA scale combined with

age, sex and ApoE genotype, could reliably predict AD severity as measured by the MMSE.

In the literature, **ApoE ε4 allele** has been linked, irrespective of the individual's clinical status or their position along the AD continuum, to elevated levels of pTau, GFAP, and NfL -BMs that have themselves been associated with a worse prognosis in neurodegenerative conditions (85,194).

Although we did not find any of such associations, in our study the possible prognostic implications of ApoEε4 allele are reflected in two observations. First, the high proportion of ApoE ε4 carriers among the AD-CVD group, particularly those classified as such solely based on extensive WMHs, which, in turn, seems to have a worse prognosis, as discussed below. Second, we observed an association between ApoEε4 carriership status and lower scores on a NPS test -the retention index. However, as noted in the Methods section, ApoE ε4 carriership status did not show any significant interaction with NPS variables in the models we constructed.

As briefly stated above**, extensive WMHs** arise as a potential prognostic BM in our study. This is based on several points.

First, we found a moderate positive correlation between PV WMHs and MTAs. These findings are in line with those by Habes et al. (201), which found correlations between greater subcortical and PV WMHs and greater brain atrophy, including within the temporal region. In turn, MTA has been widely related with worse cognitive performance and risk of progression to dementia in previous studies (202,203). In our study, both MTA and GCA showed correlations with poorer cognitive performance, but these, as mentioned, did not withstand FDR correction.

Second, WMHs and PVS have been associated with poorer cognitive performance and/or functional impairment in previous studies (171). Although it did not withstand

correction by FDR, we found a similar association -specifically, a correlation between higher PV WMHs and worse executive function.

Third, while the higher rate of conversion to dementia in the AD-CVD group aligns with previous literature reporting a worse prognosis for AD-CVD compared to AD alone (57), and aligns with both the synergistic and additive hypotheses (111), the loss of statistical significance in the sensitivity analysis suggests that this specific subgroup may have contributed disproportionately to the poorer outcomes observed in the overall AD-CVD group. This suggests that WMHs may serve as an indicator of poor prognosis, irrespective of the underlying aetiology. Regarding this latter point, future studies incorporating clinical, biological, and neuropathological cohorts of patients with positive AD BMs and extensive WMHs, but no other vascular lesions, may help clarify the extent to which CAA contributes to this phenotype.

Additionally, we observed that severe PVS in the basal ganglia were associated with poorer recognition performance, but these associations did not remain significant after FDR correction. Previous studies had found correlations between PVS located in the CSO, or both in the CSO and basal ganglia, and poorer global cognitive outcomes (204).

CAA

CAA frequency

Our study underscores the role of CAA in AD and AD-CVD, as the prevalence of CAA in both groups was high. Although our study may be subject to selection bias -given the deliberate effort to include patients with significant CVD- and our sample may not be fully representative of broader AD and AD-CVD populations, our findings are consistent with those reported Costa et al. (205). They found that, out of a memory clinic cohort comprising 622 individuals with different diagnoses (including AD (40%), VaD (16.7%)

and AD-CVD (8.2%)), two thirds exhibited non-haemorrhagic markers of CAA, half met the Boston 2.0. criteria for possible CAA, and 11% fulfilled the criteria for probable CAA.

Our results also algin with the systematic review and meta-analysis conducted by Jäkel et al. (43). They included over 2,300 individuals with AD and reported prevalence of strictly lobar CMBs (a key criterion in Boston 2.0. CAA criteria) and over 2,700 individuals with AD and reported prevalence of moderate-to-severe CAA pathology. They found that CAA prevalence based on strictly lobar CMB was 22% in the studied AD populations. In contrast, the prevalence of moderate-to-severe CAA pathology, as determined by neuropathological examination, was approximately twice as high (48%). The later aligns with the fact that although the second version of the Boston criteria (36) include non-haemorrhagic BMs - acknowledging that they tend to manifest earlier in the disease course than haemorrhagic ones- they still may fail to detect the least severe and/or early cases within the CAA spectrum.

If the prevalence of CAA in populations living with AD and/or AD-CVD as ours and previous findings suggest, CAA may substantially contribute to the clinical impact of AD and to the association between AD and CVD, positioning it as a potential therapeutic target and raising concerns about the administration of anti-amyloid therapies to these populations.

In this context, and given the significant implications of CAA, we argue that there is still a need to develop BMs that can further assist and complement Boston 2.0. criteria in the identification and accurate characterization (e.g. severity) of this pathology in vivo.

BMs indicative of CAA burden

Higher levels of sTREM2 and ucMGP were associated with lobar CMBs, a key BM of CAA. Elevated sTREM2 levels were also associated with deep CMBs -which, although possible in CAA, are more commonly related to hypertension-associated svCVD.

Additionally, as mentioned, higher sTREM2 levels were associated with severe PVS in CSO (more suggestive of CAA) and basal ganglia (more suggestive of svCVD related to traditional CVRF).

Therefore, our findings suggests that ucGMP may be a more specific CAA BM. This warrants further investigation, since, to the best of our knowledge, no associations between ucGMP and CAA had been reported before.

We found no other association between the BMs used and the CAA burden as assessed by Charidimou's score. This contrasts with the findings by Kang et al., who reported correlations between CAA and AD biofluid BMs -specifically, ptau217, GFAP and NfL- that were not entirely mediated by Aβ PET positivity (206).

Considerations regarding clinical and biological definitions of cognitive-impeding diseases, including AD-CVD

Clinical approach and definitions

Traditionally, diagnosis of cognitions impeding cognition relied on **clinical syndromes.** However, this approach has notable limitations, as it is often neither sensitive nor specific to any neuropathological condition.

With regards to sensitivity, large clinic-pathological studies in elderly cognitive unimpaired individuals- in which almost half of the participants had moderate or severe neuropathology density- showed that disorders that drive cognitive impairment- including cCVD- might remain asymptomatic for many years (108). Furthermore, not all individuals with neuropathological lesions will eventually develop the clinical symptoms associated with the underlying disease during their lifetime, and population-based studies show a divergence between the prevalence of amyloid plaques and the occurrence of dementia (207). Although some argue that if appropriate tests are used, certain degree of NPS impairment can be detected even in "subclinical" or presumptively preclinical patients

(208), this may still not apply for individuals in the earlier phases of the disease continuum.

The limited sensitivity of clinical assessments may be attributed to:

- The inherent **nature of neuropathological processes,** including the existing time lags between initial pathophysiological processes and the clinical onset of the disease. This is illustrated by the classical model of the amyloid cascade in AD (209).
- The **limited sensitivity of the methods used to evaluate cognition** in clinical practice.
- **Resilience,** which in the field of cognition refers to the brain's capacity to maintain its functions despite aging and disease, resulting in cognitive performance that exceeds expectations for the degree of underlying pathology. It is different to the related concept of "resistance", which is a system's ability to remain unaffected by harmful stimuli.

  Individual resilience factors can thus influence the point along the disease continuum in which the condition becomes clinically apparent (54).

  "Cognitive reserve"- brain's ability to solve problems and solve challenges (210)- is thought to be one of the main drivers of resilience, and can be enhanced by education and intellectual stimulation along the life course.

  Conversely, brain co-pathologies may diminish brain resilience to AD. Such role of co-pathologies is illustrated by the frequent (36.1-51.3% of a cohort of Aβ positive individuals) discrepancies between biological- assessed through PET-tau- and clinical staging (e.g.

individuals being cognitively severely impaired despite having a low tau burden) (211).

With regards to specificity, there is a big clinical overlap between diseases, as the so-called AD mimics illustrate. For instance, limbic-predominant age-related TDP-43 encephalopathy (LATE) can result in misdiagnosis of AD if the diagnosis is based solely on clinical criteria. Conversely, the same underlying pathology can manifest in diverse clinical presentations, some of which may be atypical, either in terms of frequency or in relation to specific pathological processes. The precise reasons for this phenomenon remain unclear. They may reflect a different disease trajectory, as indicated by distinct amyloid deposition patterns observed between early onset AD and late-onset AD (212). Alternatively, in some cases they may reflect the presence of co-pathology.

Therefore, and overall, considering brain co-pathologies is often key to adequately address a patient with cognitive complaints from a diagnostic perspective.

Biological approach and definitions

Although BMs have importantly contributed to a more accurate characterization of diseases affecting condition, and, in some cases, they offer great diagnostic, prognostic and therapeutic value, several factors must be considered when they are used. These include considering demographic features and comorbidities and other specificities of each individual and context and the limitations of both purely clinical and biological approaches. The most important considerations and limitations of BMs will be discussed next, building on the case of AD-CVD and reflecting on our findings.

First, and **with regards to sensitivity, cerebral MRIs** typically used in routine clinical practice may not be able to detect some of the radiological signs associated with svCVD, the leading cause of VCI, such as microinfarcts or microstructural alterations of white

matter tracts. Notably, these changes are not associated with larger infarcts in almost half of the cases (116, 213).

Second, some of the BMs we used lacked specificity. For instance, STREM2 has been associated with both vascular changes and with tau pathology, as previously noted.

As another example, emerging considerations about **WMHs and PVS –** as discussed above**-** illustrate the **specificity limitations** of many BMs. Although both WMH and PVS are well-stablished svCVD BMs, including CAA -in which WMHs and PVS may appear earlier than CMBs (214)- they are increasingly recognized as non-specific indicators of other processes such as glymphatic system dysfunction and neurovascular unit impairment.

Therefore, in individuals with positive AD BMs who exhibit extensive WMHs but no other cerebrovascular lesions, these WMHs may not reflect an associated cerebrovascular entity (e.g., svCVD due to CAA or traditional cardiovascular risk factors), but rather may result from neurovascular unit dysfunction secondary to genetic factors such as ApoE ε4 carriership (117,186,215). Whether such a case can be or not defined as an AD-CVD case depends, again, on how AD-CVD is conceptualised and understood. Notably, if the term is reserved for cases in which a distinct neurovascular entity coexists with AD, caution should be exercised when classifying an individual as having AD-CVD based solely on the presence of extensive WMHs, as these do not necessarily indicate underlying svCVD, but may reflect alternative -although often related- mechanisms, including genetic factors. Although not reflected by our findings, it is possible that the same applies to severe PVS.

However, in our opinion, some of the currently used diagnostic frameworks (Table 2) do not adequately account for all these considerations. For instance, according to the VASCOG-2 criteria, individuals with extensive WMHs are categorised as having a significant CVD. Likely, the presence of extensive WMHs precluded the diagnosis of

“probable AD” according to older criteria frameworks, such as NIA-AA 2011 (23). While the 2024 NIA-AA AD diagnosis and staging criteria (172) represent a significant improvement -more clearly recognizing the possibility of co-pathology and explicitly including WMHs as a vascular BM (172)- we believe further refinements are still needed.

The WMHs distribution patterns may help orientating the underlying pathological processes, since different patterns and distributions of WMHs have been observed among AD, VaD and AD-CVD (216,217) and among these groups and other dementias like PD and LBD (218). In AD, such pattern is characterized by a greater involvement of the bilateral PV and parietal lobe regions (119,219). Rennie et al. (218) found that the percentage of individuals with a high WMH load (Fazekas 2 or 3) was higher in the VaD (62%) and the LBD (43%) groups than in the AD cohort.

Third, **BMs just reflect the presence of an underlying pathology** but often provide little information about the clinical impact this pathology is having, that is, the contribution it is having to patients’ clinical phenotype. In other words, their positive predictive value for cognitive impairment is not straight forward, as it depends on many factors, including mechanisms of resistance and resilience (as discussed above), the overall lesion burden, their specific location within the brain, and the presence or absence of co-pathologies. The other way round, clinical status -as stated above- is a poor indicator of the underlying pathophysiological processes. All of this is illustrated by the difficulty in evaluating the clinical impact that cCVD has over cognition, as discussed.

Fourth, to accurately interpret BMs, it is essential to also consider **pre-analytical factors and the variability in BMs values both between and within individuals**.

Preanalytical factors include the methods of sample collection, handling, and processing, and can significantly influence BM levels. Currently, several techniques are employed to measure fluid BM concentrations, including SIMOA, mass spectrometry-based detection (MDS), immunomagnetic reduction, and electrochemiluminescence,

each with distinct characteristics and limitations that must be accounted for. In this regard, standardization is crucial, particularly for blood-based BMs. Notably, we took all of these into consideration with the invaluable help of the collaborating authors working in the biochemistry department of VH.

The variability in BMs levels between and within individuals depends on various factors, both disease- and non-disease-associated, that can influence their expression or concentrations. These factors include age, body mass index, race and ethnicity, comorbidities, and genetic factors. With regards to comorbidities, studies involving community-based cohorts have demonstrated that levels of key AD BMs are notably higher in individuals with comorbid conditions such as myocardial infarction and chronic kidney disease, particularly in cases of moderate to severe kidney disease (220,221). While the impact of comorbidities on BM levels is particularly pronounced in blood-based BMs, some comorbidities can also affect CSF BMs. For example, in idiopathic normal pressure hydrocephalus, CSF amyloid and tau levels vary depending on the location of collection (intraventricular vs. lumbar) and the timing of the harvesting (222). Beyond their potential to influence BM levels, considering comorbidities is also critical for an accurate interpretation of BMs within the individual's specific clinical context. In fact, it has been stated that the effect of comorbidities on AD may be as significant as the individual's amyloid status (129).

Variability in BMs levels between and within individuals is particularly pronounced in **blood-based BMs**, as additional factors come into play, like CSF-to-blood clearance, peripheral sources of pathological proteins used as BMs, and blood volume and constituents -such as proteins to which BMs may attach (223).

One of the main disease-associated factors is the **dynamism** of many BMs. BMs change over time, likely reflecting the evolving nature of neurodegenerative and vascular diseases. As mentioned in the corresponding section, the “svCVD picture” of a given individual is dynamic, which may hinder diagnostic evaluations (e.g., diagnosing a

delayed onset VCI may be difficult). Conversely, the dynamism of some BMs may be of help in determining prognosis. For instance**,** blood pTau217 steadily increase as the AD progresses, and higher levels of pTau217 predict the conversion from cognitively unimpaired to MCI, or from MCI to AD dementia, in patients with a positive cerebral amyloid PET (72). Such dynamism may be also used to monitor the response to treatments.

Limitations

W**e acknowledge several limitations of the present study.**

Although statistical generalization is assumed, the study was based on a non-random, convenience sample. Furthermore, we made a specific effort to recruit patients with a significant CVD. However, the proportion of AD-CVD over the whole sample is consistent with that reported in clinical-pathological studies (16,17), and the patients who met screening eligibility criteria but did not meet enrolment criteria (e.g. those who underwent the lumbar puncture but had negative or inconclusive CSF AD BMs) did not differ significantly in clinical or demographic characteristics, except for the group with normal CSF AD BMs being younger than those in the AD group. Therefore, our sample may be representative of the patients with MCI or mild dementia being cared for at our hospital. Moreover, this approach was deemed the most feasible way of conducting a study with carefully selected individuals with AD-CVD.

Additionally, the relatively small sample size may have limited our ability to detect some significant differences or stronger associations.

We did not include patients with AF, as usually anticoagulation is prescribed for this condition, and it was deemed inappropriate to withdraw anticoagulant therapy for

performing a lumbar punction. However, since AF is the main cause of cardioembolic CVD in elderly patients, this decision may have limited the generalisability of our findings to the AD-CVD population without CVD of cardioembolic origin.

The NPS protocol was based on routine clinical practice and was not specifically designed for this study. As a result, not all cognitive domains were comprehensively assessed in all participants. However, the use of a clinically grounded assessment may enhance the generalizability of our findings to real-world settings.

While the AD-related BMs we employed had been previously validated, those intended to serve as proxies for cerebrovascular processes -such as ucGMP and sTREM2- had not been validated at the time the study was conducted. Therefore, our findings should be interpreted as exploratory in this sense.

We specifically assessed that there were not significant differences between AD and AD-CVD in the distribution of various factors known to influence BM levels, as discussed above. For example, we confirmed that comorbidities did not differ significantly between groups. However, as in most studies on this topic, we did not account for all potential sources of variability. For instance, we assessed imaging and biofluid BMs cross-sectionally, so we did not consider the dynamism of many BMs. As another example, we assessed ApoE genotype as a genetic factor. However, other genetic factors which are known to play a role in AD and CVD (224,225) were not assessed.

Another limitation is that the control group lacked AD BMs aside from blood-based BMs, which were neither established in clinical practice nor validated at the time the blood samples were obtained.

Given that we used VASCOG-2 criteria to classify participants, it is likely that we have misclassified some cases of AD with extensive WMHs as AD-CVD. Specifically, 31.9% of the individuals classified as AD-CVD in our study were classified as such based solely on extensive WMHs, that is, without having any other significant cerebrovascular lesions.

This potential misclassification is supported by the fact that some of the differences we found between AD and AD-CVD only became statistically significant in the sensitivity analysis. Similarly, several BMs improved their discriminative capacity between AD and AD-CVD in such analysis. On the other hand, we may have misclassified as pure AD participants who had svCVD that we were unable to detect.

The criteria for AD diagnosis based on CSF BMs followed the guidelines in force during the study period. However, updated criteria were published shortly after the end of the recruitment phase (172). Although, as stated, the participants classified as having AD in our study (with or without significant CVD) would still meet AD criteria under the new guidelines, it is possible that some individuals previously classified as having inconclusive CSF AD profiles would now be categorized as having AD (e.g., low Aβ42 with normal p-Tau and t-Tau, but pathological p-Tau/Aβ42 and t-Tau/Aβ42 ratios).

Although all individuals in our study presented with cognitive impairment, our assessment of clinical progression may not have been sensitive enough to detect subtle cognitive changes. We acknowledge that evaluating clinical progression in neurodegenerative conditions often require longer follow-up periods and/or more sensitive assessment tools than those employed in our study -MMSE and GDS. Consequently, our capability to identify BMs with prognostic value has been limited. In this context, follow-up NPS assessments for all participants would have strengthened the study.

Second part of the discussion (study two)

This work has aimed to contribute to the existing body of knowledge regarding AP and health through exploring how AP influences two areas where existing evidence is less robust: cognition and cCVD. It has sought to do so in the belief that constructing and

appropriately disseminating high-quality evidence supporting the relationship between AP and adverse health outcomes may help elevate the issue on the agenda of relevant stakeholders and support the formulation and implementation of appropriate policies.

In our study, the significant correlations observed among all the APs analysed suggest a shared source for all or most of them, most likely urban road traffic, given the nature of the pollutants and the urban setting of the study.

General characteristics of the cohort

Hypertension is highly prevalent among adults in the age group of the cohort (median age was 64 (IQR 54-67) years) with rates increasing in many countries. In Spain, the prevalence of hypertension in the population aged 30-79 years old was 33% in 2019 (226). Although prevalence rates vary by region, similar figures have been reported in many other countries (227). Based on this, we argue studying the effect that exposure to AP has on hypertense individuals is relevant.

On the other hand, hypertension was reasonably well controlled among participants, suggesting that the observed findings related to cCVD progression cannot be fully attributed to inadequate hypertension management. The prevalence of other traditional CVRF was relatively low with 28.2% for dyslipidaemia, 23.5% for diabetes, and 15.2% for active smoking. The prevalence of AF was particularly low (3.9%). The median REGICOR score was 5 (IQR: 4,7), which is consistent with a low to moderate global cardiovascular risk.

Correlations between AP and clinical variables

Acknowledging that exposure to AP can worsen blood pressure control (159), we explored potential associations between key CVRF and conditions and various APs. We

identified four statistically significant, albeit weak, correlations: higher levels of PM2.5 were associated with AF and dyslipidaemia; higher levels of PM_{coarse} were associated with increased DBP; and higher levels of NO2 were associated with lower DBP. Given the high number of comparisons made, these associations might have been casual. Nonetheless, some of these results are consistent with prior studies linking AP exposure to AF, hypertension and diabetes (228). Further, they highlight areas that warrant further investigation. For example, consistently linking $PM_{2.5}$ with AF -a major cause of stroke- would be a significant and clinically relevant finding.

AP and cCVD

Our study demonstrated that exposure to various APs is associated with the presence and mid-term progression of cCVD in individuals without a prior diagnosis of CVD. Therefore, our first hypothesis concerning Study Two was confirmed.

These findings align well with previous research, including meta-analysis, which have reported associations between short- and long-term exposure to AP and increased risk of stroke (229) and stroke-related mortality (230). Associations between exposure to AP and an increased risk of CBI were also found on almost one and a half million MRIs performed in health screening centres in China (231).

The prevalence of CBI in the cohort of the study -the ISSYS cohort- had already been published by Delgado et al. (232). Such prevalence was associated with $PM_{2.5.}$ In turn, exposure to NO2 was that associated to the presence of extensive WMHs in both periventricular and subcortical locations at baseline. $PM_{2.5.\ absorbance}$ was also associated with the latter.

The proportion of participants exhibiting the most common form of cerebrovascular change progression -marked WMHs progression- was lower (19.7%) to that reported by in the studies by Ammirati et al (69) and Jiang et al. (68) already commented in the introduction (49% and 50.2% correspondingly). These differences could be attributed, at

least in part, to a higher baseline burden of WMHs -56% for Jiang et al. (not specified for individuals in Ammirati et al.) vs 44% in our study- which was identified in both studies as the most important predictor of WMH progression. Alternatively, the differences could be due to a higher cardiovascular risk compared to the age- and sex-matched participants in the ISSYS cohort. This higher cardiovascular risk is presumed from the fact that, in contrast to the participants in the ISSYS cohort, participants in the cited studies had either had a minor stroke or had been derived for a carotid arteries ultrasound for clinical (non-specified in the article) reasons.

Exposure to NO2 showed the strongest association with progression in our study, as it was associated to the progression of extensive WMHs and to the combined progression score. Exposure to NO_x more broadly was also associated with the later, as well as with the incidence of CMBs.

The finding that exposure to NO_2 or NO_x was equivalent to ten years of aging in terms of cerebrovascular lesions aligns with the well-documented inequities in average life expectancy among neighbourhoods with differing built environments. These inequities are largely shaped by existing urban and environmental policies and that are often related with socioeconomic factors, and that are deeply interrelated with exposure to AP (233).

Although our study provides new evidence with regards the association AP and cCVD, this warrants further exploration to address the limitations of this study -detailed below- and to investigate whether our results are replicated in other settings.

AP and cognition

The proportion of MCI among adults over 50 years of age varies widely depending on population characteristics and diagnostic criteria, ranging from 3 to 87%, although most

studies report prevalence rates of approximately up to 20% (234,235). This is consistent with the prevalence observed in our cohort (7.1%). MCI is a very loose and diagnostic category which can be caused by a very heterogeneous group of conditions. While some of these are neurodegenerative, many of these are not (e.g., primary mood disorders, sleep deprivation, etc.), as illustrated by the fact that, in our sample, 21 individuals with MCI regressed to normal cognition during follow-up.

In previous studies, AP has been linked with lower cognitive function both globally and across specific cognitive domains (236,237), cognitive impairment throughout the lifespan (236,237), neuropsychiatric symptoms in cognitive impairment (238), clinical syndromes like MCI and dementia (154), and BMs of neurodegeneration and AD (239,240). A recent systematic review concluded that the association between AP and lower cognition across the lifespan is robust (236).

We aimed to contribute to this growing body of evidence by studying the relationship between cognitive function and AP in a cohort characterized by a particularly high risk of having and developing a cCVD – a population, to the best of our knowledge, that had not been previously studied in this regard.

However, in our study we did not find any association between AP and cognition. Therefore, our second hypothesis concerning Study Two was not confirmed. As in Study One, this may reflect a Type II error, which in turn may be due to limitations in the sensitivity of the cognitive assessment tools used or in the study design itself, which may not have been sufficiently robust, long-term, or statistically powerful to detect subtle cognitive changes associated with AP and its underlying mechanisms (e.g., CVD).

Implications and relevance

To the best of our knowledge, this is one of the few studies assessing the relationship between AP and cCVD, and the first to examine the association between AP and CVD

progression over time. As mentioned, the study was performed in a population at elevated risk for CVD. Furthermore, all the most important APs were analysed and a well-established method to estimate the exposure to them was used. We adjusted the cognitive outcomes by cCVD presence and progression, which is not usually considered.

A notable point to highlight is that all the analysed APs in our study are above the recommended maximum levels established by WHO (World Health Organization) and the EU (Table 14). A Chinese study reported similar levels of APs ($PM_{2.5}$ 15.61 µg/m3, PM_{10} 30.08 µg/m3, NO2 35.98 µg/m3), while in a European one their mean levels varied significantly between the 11 cohorts included ($PM_{2.5}$ 8-19, PM_{10} 14-46, NO2 15-60) (231,241). It is worth pointing out that despite AP levels in LMIC are higher, most of these type of studies have been performed in HIC (236).

Our findings underscore the need to reduce urban road traffic, as NO2 and $PM_{2.5\ absorbance}$ -both primarily emitted by road traffic in urban environments- were among the pollutants most strongly associated with cCVD. While the results are not conclusive, and must be interpreted considering the limitations discussed below, we believe that, in accordance with the precautionary principle, these findings provide sufficient to support further research and to inform the implementation of policies aimed at reducing exposure to AP.

Difficulties and limitations (Study Two)

Some of the limitations and difficulties of Study Two are related to **the nature of the phenomena under study**, especially cognition and AP.

To estimate the exposure to AP in an individual basis is difficult. For instance, although we made sure that the usual residence had not changed throughout the study, we did not consider the workplaces of the participants nor their mode of transport to them. Moreover, acknowledging that exposure to AP might have lag effects on health, usual residence locations prior to the beginning of the study were not assessed in all the

participants. In the analysis, we did not consider some factors that may have acted as confounding factors, such as exposure to environmental noise or the economic level of the participants.

Further, there is an important heterogeneity in the methods used for measuring exposure to AP. Heterogeneity is present in other methodological aspects of studies addressing this topic, such as the main outcomes or the included variables, thus contributing to inconclusive or conflicting results in the literature.

In the same line, it is likely that a complex, multifactorial model underlies the association between AP and many adverse health outcomes. The degree in which AP contributes to them might be discrete.

A longer follow-up period and/or more sensitive tools would have been necessary to assess cognition, especially considering that the prevalence of cognitive impairment among healthy individuals aged 50–70 years is typically no higher than 10–20% (235).

Additional limitations may stem from aspects of the **study design**. Specifically, there is a potential risk of selection bias, as participants with existing cerebrovascular lesions on baseline MRI were prioritized for follow-up. While this approach provides valuable insights into the effects of AP exposure on a particularly vulnerable population - individuals with cCVD- it also limits the generalisability of our findings. In particular, the conclusions regarding the progression of cCVD in relation to AP exposure may apply primarily to a narrowly defined subgroup: hypertensive individuals aged 50 to 70 living in Barcelona with cCVD.

Although AP levels are known to be comparable across several EU cities (242), our study was conducted in a specific population within a particular geographic and temporal context. Therefore, caution is needed when extrapolating these findings to other settings

or periods. In this sense, we encourage to reproduce similar studies in other locations and with more diverse and inclusive cohorts -prioritising areas in which AP is especially problematic (e.g. large urban settlements in LMIC). Paradoxically, these are often the regions where such research remains scarce.

In our study, we found almost no significant associations between CVRF and APs, suggesting that these are unlikely to have acted as major confounding factors. However, it would have been interesting to include data on participants' ApoE genotype, as this is an important risk factor for CAA-CVD. However, this data was unfortunately not available.

It is also important to acknowledge the potential impact of conducting multiple analyses, which may have increased the risk of Type I errors. Additionally, the assessment of cCVD progression was based solely on qualitative measures.

Importantly, correlation is not causation, so it cannot be presumed from our study that there is a causal relationship between exposure to some of the studied APs and the prevalence and incidence of cerebrovascular lesions. However, as stated, we believe these findings underscore the need for further research to explore this topic in greater depth.

Lastly, we did not compare exposure to AP or its effects across different subtypes of MCI - such as vascular vs. neurodegenerative or amnestic vs. non-amnestic- due to the limited number of participants with MCI at baseline. This remains an interesting area for future research, acknowledging that different types of MCI are related to different underlying aetiologies (235).

CONCLUSIONS

1. We would like to highlight the following observations regarding AD and AD-CVD characterization:
    a. A certain degree of co-pathology is present in most participants, including those classified as having “pure” AD. Rather than as distinct entities, AD and AD-CVD likely exist along a shared neurodegenerative-neurovascular continuum, where the clinical presentation of AD-CVD is, in many cases, primarily driven by AD pathology.
    b. Individuals should not be classified as having AD-CVD based solely on the presence of WMHs, as these do not necessarily indicate underlying svCVD, but may reflect alternative -although often related- mechanisms, including genetic factors.
    c. The prevalence of ApoE ε4 carriership status is high among individuals with either AD or AD-CVD, particularly among those with extensive WMHs or a first-degree family history of dementia. ApoE ε4 may also be associated with a lower cognitive performance.
    d. sTREM2 may serve as a valuable -though non-specific- BM of svCVD, whereas ucGMP should be further studied as a potential BM of CAA.

2. AD-CVD may have a worse prognosis than “pure” AD cases. However, this finding may be partly driven by the potential misclassification of participants in the AD-CVD group based solely on the presence of extensive WMHs.

3. ucGMP, sTREM2, and t-tau appear to be promising BMs for differentiating individuals along the AD-CVD continuum -from those closer to the “pure AD” end

from those closer to the “pure VCI” end. T-tau may be useful for assessing the neurodegenerative burden in cases of AD-CVD.

4. Extensive WMHs are associated with greater MTA and may contribute to the worse clinical outcomes observed among those classified as AD-CVD.

5. CAA is a frequent co-pathology across the entire AD-CVD continuum, possibly including cases that lie closer to the “pure AD” end.
   In our study, no BM showed a significant correlation with CAA burden or severity.

6. Exposure to several APs was predictive of the presence and accumulation of cCVD, suggesting that AP may represent a modifiable risk factor for CVD in individuals already predisposed to it. Specifically:
   a. At baseline:
      i. $PM_{2.5}$ exposure was associated with higher odds of CBI.
      ii. NO_2 exposure was associated with higher odds of extensive subcortical and periventricular WMHs.
      iii. $PM_{2.5absorbance}$ exposure was associated with higher odds of extensive subcortical WMHs
   b. Over time, exposure to:
      i. NO_x exposure was associated with higher odds of developing CMBs and a higher combined progression score.
      ii. NO_2 exposure was associated with marked progression of subcortical WMHs and a higher combined progression score.

7. Exposure to AP has not been associated with worse cognition in our study, but existing evidence suggests a potential link that warrants further investigation.

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Appendix 1

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ORIGINAL ARTICLE

Ambient air pollution, covert cerebrovascular disease and cognition: results from the ISSYS study

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Abstract

Background and purpose: Although air pollution (AP) has been associated with stroke and dementia, data regarding its relationship with covert cerebrovascular disease (cCVD) and cognition over time are sparse. The aim of this study was to explore these relationships.

Methods: A prospective population-based study of 976 stroke-free and non-demented individuals living in Barcelona, Spain, was conducted during 2010–2016. A land use regression model was used to estimate the exposure of each participant to AP: NO_x, NO_2, $PM_{2.5}$, PM_{10}, PM_{coarse} and $PM_{2.5}$ absorbance. Cognitive function and cCVD were assessed at baseline ($n=976$) and 4 years after ($n=317$). Multivariate-adjusted models were developed.

Results: At baseline, 99 participants (10.1%) had covert brain infarcts and 91 (9.3%) had extensive periventricular white matter hyperintensities (WMHs). Marked subcortical WMH progression was seen in 19.7%; the incidence of other covert cerebrovascular lessons ranged between 5% and 6% each. $PM_{2.5}$ was related to higher odds of having a covert brain infarct (odds ratio [OR] 2.21; 95% confidence interval [CI] 1.06–4.60). $PM_{2.5}$ absorbance was related to higher odds of having extensive subcortical WMHs (OR 1.72; 95% CI 1.13–2.60), whereas NO_2 was related to higher odds of having extensive subcortical (OR 1.66; 95% CI 1.17–2.35) or periventricular (OR 1.96; 95% CI 1.10–3.50) WMHs and to higher odds of developing marked subcortical WMH progression (OR 1.40; 95% CI 1.05–1.90). NO_x was related to incident cerebral microbleeds (OR 1.36; 95% CI 1.04–1.79). There was no association between AP and cognition.