

Olfactory assessment and nasal brushing to investigate mechanisms of neurodegeneration and aging

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ABSTRACT

The olfactory system remains largely underexplored in biomedical research, despite its functional richness, high plasticity, and unique accessibility in humans. As both a sensory interface and a source of easily accessible human tissue, it represents a relevant model for investigating early biological processes at both functional and cellular levels.

The first part of this thesis focuses on Alzheimer's disease and aims to characterize the progression of tau pathology within the olfactory system. Using a cohort covering the clinical continuum of Alzheimer's disease, the results show that tau pathology follows a retrograde propagation along olfactory pathways, spreading from central brain regions toward peripheral structures. These findings support the value of olfactory testing as a complementary functional tool to improve early diagnosis of Alzheimer's disease. In contrast, the analysis of the olfactory neuroepithelium using nasal brushing appears to be of limited relevance for the direct detection of tau pathology, as pathological burden seems to occur at a late stage in this tissue.

Beyond neurodegeneration, the olfactory system also offers opportunities as a source of human tissue for the study of biological aging. The second part of this work therefore focuses on the olfactory neuroepithelium, which can be accessed rapidly and non-invasively by nasal brushing, to investigate cellular mechanisms associated with aging.

Analysis of a cohort including healthy individuals across a wide age range, as well as patients with telomere biology disorders, reveals an association between proteostasis dysfunction and the initiation of cellular senescence, independently of telomere attrition.

Overall, this work highlights the value of olfactory-based tools, used either as functional approaches or as sources of human tissue, for both fundamental research and clinical applications.

LIST OF ABBREVIATIONS

A		
<i>Aβ</i>		Amyloid Beta peptides
<i>AD</i>		Alzheimer's Disease
<i>AON</i>		Anterior Olfactory Nucleus
<i>APP</i>		Amyloid Protein Precursor
C		
<i>CSF</i>		Cerebrospinal Fluid
<i>CTF</i>		Carboxy-Terminal Fragment
<i>CTR</i>		Carboxy-Terminal Region
E		
<i>EC</i>		Entorhinal Cortex
M		
<i>MCI</i>		Mild Cognitive Impairment
<i>MRI</i>		Magnetic Resonance Imaging
<i>MTBR</i>		Microtubule-Binding Region
N		
<i>ND</i>		Neurodegeneration
<i>NDD</i>		Neurodegenerative Disease
<i>NFT(s)</i>		Neurofibrillary tangle(s)
<i>NTR</i>		Amino-Terminal Region
O		
<i>OB</i>		Olfactory Bulb
<i>OC</i>		Olfactory Cortex
<i>ONE</i>		Olfactory Neuroepithelium
P		
<i>PET</i>		Positron Emission Tomography
<i>PRR</i>		Proline-Rich Region
<i>PTMs</i>		Post-translational modifications
R		
<i>ROS</i>		Reactive Oxygen Species
S		
<i>SA-β-gal</i>		Senescence associated β-galactosidase
T		
<i>TBD</i>		Telomere Biology Disorders
<i>TIF</i>		Telomere dysfunction-Induced Foci

GENERAL INTRODUCTION

INTRODUCTION

CHAPTER 1

I. Alzheimer's Disease: from pathological mechanisms to diagnostic challenges

1. Conceptualization of Alzheimer's Disease

1.1. Public health issues

Alzheimer's disease (AD) was first described in 1906 by the German neurologist Alois Alzheimer, who provided the clinical and neuropathological report through his observation of Auguste Deter, a patient suffering from early-onset dementia [1-3]. Since then, it has become sadly recognized as the most common form of dementia worldwide. It is characterized by a progressive and irreversible course, marked by the accumulation of specific brain lesions, which are responsible for cognitive and functional decline [4]. Even today, more than a century after its initial description, AD remains a major neurodegenerative condition at the center of extensive clinical and fundamental research, due to its societal impact.

In 2025, AD represents a global public health challenge, with a prevalence that increases sharply with age. It is estimated to affect about 10% of people aged 65 and over, and between 30% and 35% of those over 80. In Belgium, the incidence is estimated at nearly 20,000 new cases per year, confirming the growing importance of this disease in an aging population. This distribution is not uniform, as a marked sex difference is observed: women account for approximately two-thirds of diagnosed cases which is around 66%. In some contexts, such as advanced age, the percentage of female cases can be more than double that of men [5]. These steadily rising figures highlight the magnitude of the issue, both from health and socio-economic perspectives. The consequences include a significant financial burden and an increased demand for medical and social resources. Globally, the projections are particularly alarming: by 2050, the number of affected individuals could reach around 131 million, more than double the current figures, confirming AD as a true global burden for healthcare systems and societies as a whole [6].

1.2. Clinical manifestations

The evolution of AD is classically described as progressive, irreversible, and closely linked to pathophysiological mechanisms that begin long before the appearance of the first clinical symptoms [7-10]. Although this progression is influenced by various factors such as overall health, the presence of comorbidities, or the age at diagnosis, it is generally understood as a continuous process consisting of three distinct phases (**Figure 1**).

The first phase, known as the preclinical phase, may last up to two decades [8-10]. It is characterized by the gradual development of cerebral pathophysiological abnormalities, even as individuals maintain normal cognitive and functional performance [8, 10]. This silent and asymptomatic stage explains why affected individuals do not seek medical attention and are generally unaware of the ongoing changes. Detection at this stage usually occurs only incidentally, particularly during clinical studies or medical examinations conducted for other reasons. The main hypothesis explaining this clinical invisibility is that, at this stage, the cortical lesions present are not yet abundant enough to impair brain function, thanks to still-effective compensatory mechanisms [10]. When these mechanisms become exhausted, the disease progresses into its second phase.

This second phase, commonly referred to as the prodromal phase, corresponds to the emergence of the first detectable symptoms [11]. Although episodic memory impairment often constitutes the most prominent initial complaint, other manifestations may also appear [12]. Language difficulties (such as anomia, reduced vocabulary, and naming errors), attention deficits, and alterations in executive functions such as planning, cognitive flexibility, or problem-solving, are frequently observed.

These signs remain mild at first but mark a change from previous functioning and start to interfere with daily activities [9, 10]. Temporal disorientation, mistakes in managing complex tasks (such as finances or travel planning), and a reduced ability to learn new information may

also occur. At this stage, the disease becomes clinically identifiable, as patients themselves or their relatives notice these difficulties and seek medical evaluation.

The third phase is the dementia phase, which marks a clear decline in cognitive and functional abilities. It is not a uniform state, but a continuum typically divided into three stages: mild, moderate, and severe [6, 9, 10].

In mild dementia, patients exhibit more pronounced cognitive deficits than in the prodromal phase, with severely impaired episodic memory, marked temporal disorientation, and a reduced ability to perform complex activities such as maintaining employment. Some degree of autonomy may still be preserved, but it is fragile and often requires supervision.

In moderate dementia, the loss of autonomy becomes evident. Patients face increasing difficulties with daily activities such as meal preparation, personal hygiene, or dressing. Spatial disorientation intensifies, exposing patients to risks of wandering or getting lost even in familiar environments. Language and communication disorders worsen, limiting social interactions. This stage is also marked by the emergence of significant behavioral disturbances that are often challenging to manage.

Finally, severe dementia is characterized by almost complete dependence on caregivers. Patients gradually lose the ability to recognize loved ones, to walk, or even to feed themselves. Motor functions and basic reflexes may be affected, leading to a bedridden state. Associated medical complications such as respiratory infections (notably aspiration pneumonia related to swallowing difficulties), pressure ulcers, or malnutrition, have become common causes of morbidity and mortality [13].

Thus, this terminal stage of AD corresponds to a gradual but inexorable reduction in life expectancy. It highlights both the profound

individual burden on the patient and the major impact on healthcare systems.

Beyond cognitive decline, patients may also exhibit a wide range of behavioral and psychological disturbances. These manifestations may occur at different stages of the disease. Among the early symptoms, apathy, irritability, anxiety, and depressive symptoms are frequently observed and contribute to diminished quality of life from the earliest clinical stages [14, 15]. As the disease progresses, more severe manifestations may emerge, such as agitation, wandering, verbal or physical aggression, sleep disturbances, and hallucinations or delusions, which are more common in the advanced stages [16-18].

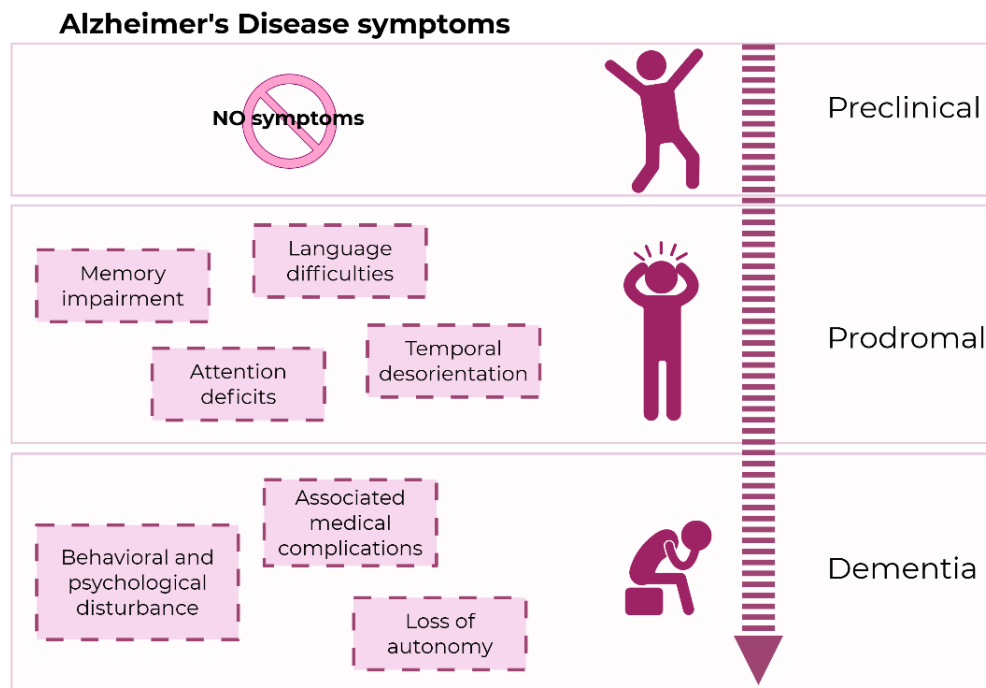


Figure 1. Alzheimer's Disease symptoms progression. Alzheimer's disease follows a gradual progression, beginning with an asymptomatic preclinical phase. This is followed by a

prodromal phase marked by the onset of cognitive symptoms, which intensify in the dementia stage and result in substantial loss of autonomy in everyday life. Generated by biorender.

1.3. Risk factors

Among the elements that strongly influence the onset and progression of the disease, age remains the principal risk factor. However, as the disease is multifactorial, many other risk factors have been identified.

Lifestyle factors such as sedentary behavior, lack of regular exercise, poor diet, and chronic smoking significantly increase the risk of developing the disease. These harmful habits notably contribute to the higher incidence of cardiovascular diseases and diabetes which are two conditions themselves associated with an increased risk of cognitive decline [19-21]. Conversely, a healthy lifestyle, characterized by a balanced diet, regular physical exercise, good sleep quality, and sustained cognitive stimulation, appears to exert a protective effect [22, 23]. Taken together, these environmental and behavioral risk factors can increase the likelihood of developing AD by 25% to 40% [24].

Besides these factors, certain genetic determinants have also been identified. Several susceptibility genes are linked to a higher risk of developing the sporadic form of the disease. Among these, the apolipoprotein E gene (ApoE) is the most extensively studied to date. It encodes a protein involved in lipid transport and cerebral cholesterol metabolism [25]. Three main alleles have been identified: *ApoE2*, *ApoE3*, and *ApoE4*.

The *ApoE4* allele, particularly when present in the homozygous state, constitutes a major risk factor for AD by promoting amyloid- β aggregation and deposition, impairing its clearance, and exacerbating neuroinflammation and neuronal damage. Heterozygous carriers present an elevated risk, while homozygous carriers have a risk increased by a factor of 10 to 15 compared with non-carriers [26]. Approximately 15% of affected individuals are homozygous for *ApoE4*,

confirming the importance of this genetic factor in the general population. However, the presence of *ApoE4* allele does not inevitably lead to the development of the disease; it is considered a susceptibility factor rather than a causal gene. Conversely, the *ApoE2* allele seems to exert a protective effect, reducing the risk of developing the disease by nearly half. In the Belgian non-demented population, ApoE3 is the most common isoform, while ApoE2 is less frequent and ApoE4 is present in a minority of individuals [27].

Beyond *ApoE*, several other risk genes have been found through genome-wide association studies (GWAS). One of the most consistent is the clusterin gene (*CLU*), which helps remove amyloid peptides and regulates the inflammatory response [21]. Another gene, triggering receptor expressed on myeloid cells 2 (*TREM2*), highlights the important role of neuroinflammation in the disease. Although the effects of these genes are smaller than that of *ApoE4*, their discovery shows that the sporadic form of AD is multifactorial and polygenic, resulting from complex interactions between genetic and environmental factors [20, 21].

1.4. Autosomal dominant Alzheimer's Disease

In most cases, which is approximately 99%, AD occurs in a sporadic form. However, in rare instances (about 1% of cases), it can be attributed to genetic mutations responsible for early onset familial forms, which manifest before the age of 65 [28].

These hereditary forms are mainly linked to mutations in the presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes, both of which are involved in the γ -secretase enzymatic complex. They are associated with an exceptionally early onset of symptoms, sometimes appearing as early as the third or fourth decade of life [29, 30].

Even more rarely, mutations in the gene encoding the amyloid precursor protein (*APP*) have been identified [31]. These mutations disrupt the normal processing of the protein, promoting the pathological accumulation of amyloid peptides. Such alterations lead

to the formation of amyloid plaques, which, by accumulating within brain tissue, constitute one of the major neuropathological hallmarks responsible for the development and progression of AD.

2. Alzheimer's Disease neuropathological signatures

Two characteristic types of neuropathological lesions are responsible for AD: amyloid plaques and neurofibrillary tangles (NFTs) [32]. These abnormalities progressively invade different brain regions, leading to neuronal degeneration and disruption of synaptic connections. This progressive loss of neuronal networks constitutes the major biological substrate underlying the clinical manifestations observed throughout the course of the disease [33]. From a chronological perspective, the accumulation of amyloid peptides in the cortex generally precedes the appearance of NFTs.

2.1. Amyloid pathology

2.1.1. Physiological role of APP

The amyloid precursor protein (APP) is a transmembrane glycoprotein encoded by a gene located on chromosome 21 [34]. Through alternative splicing mechanisms, this gene generates several mRNA transcripts, leading to the synthesis of protein isoforms of varying lengths, ranging from 365 to 770 amino acids [31, 35]. APP is described as a ubiquitous protein, as its different isoforms are expressed in numerous peripheral tissues, although it is particularly abundant in brain tissue [36]. In neurons, APP is transported along axons to synaptic terminals, where it undergoes cleavage and secretion [37].

Functionally, APP plays an essential role in several physiological processes within the central nervous system. It is involved in neurite outgrowth, as well as in the formation and stabilization of synapses, thereby directly contributing to neuronal plasticity [38]. APP also exerts a neuroprotective function by regulating certain intracellular signaling cascades. For example, it modulates the activity of cyclin-dependent kinase 5 (Cdk5), which helps limit the pathological hyperphosphorylation of the tau protein.

Studies conducted on APP-deficient animal models have confirmed these observations: the absence of APP leads to abnormalities in synaptic transmission, such as impaired long-term potentiation (LTP) or altered GABAergic responses, as well as disturbances in cholinergic transmission. These findings highlight the crucial importance of APP in maintaining synaptic efficiency and neuronal physiology [39-41].

2.1.2. Proteolytic metabolism and cleavage of APP

The proteolytic metabolism of APP can follow two main pathways: the non-amyloidogenic pathway, which is considered physiological and protective, and the amyloidogenic pathway, which underlies pathological processes [35] (**Figure 2**).

In the non-amyloidogenic pathway, APP is cleaved by α -secretase within the sequence corresponding to the A β peptide, thereby preventing its formation [42]. This cleavage produces soluble APP α , a fragment with neuroprotective properties that promotes neuronal survival and synaptic plasticity, as well as a membrane-bound fragment known as the C-terminal fragment generated by α -secretase cleavage, α CTF. In a second step, γ -secretase acts on this fragment to release two products: the small extracellular peptide P3, which is non-toxic and lacks amyloidogenic potential, and the APP intracellular

domain (AICD), which is involved in transcriptional regulation and nuclear signaling.

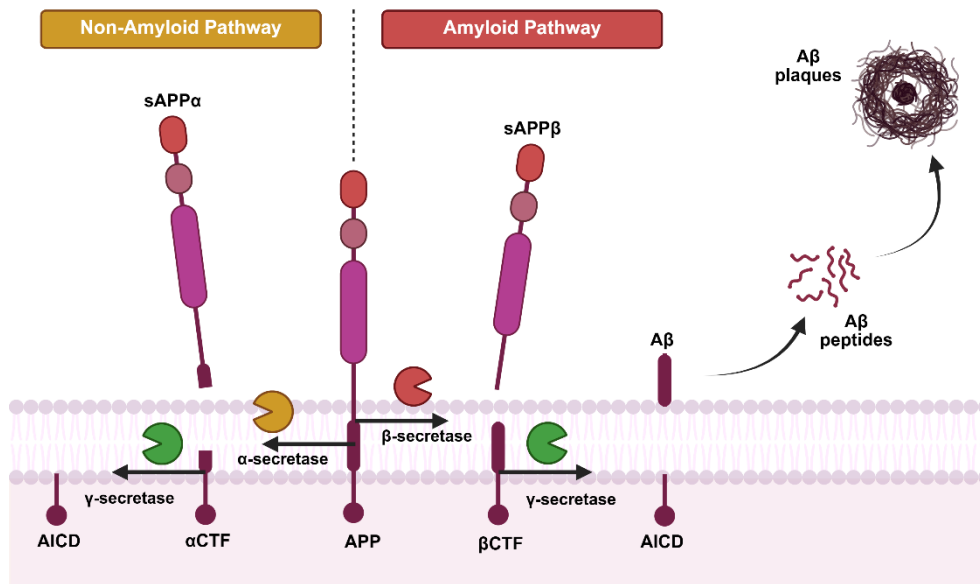


Figure 2. APP cleavage pathways and A β plaques formation. APP is processed through two pathways. The non-amyloidogenic pathway involves α - and γ -secretase cleavage and prevents A β formation. The amyloidogenic pathway involves β - and γ -secretase cleavage and leads to the production of A β peptides, including A β 40 and the more aggregation-prone A β 42. Generated by biorender.

In contrast, the amyloidogenic pathway begins with the cleavage of APP by β -secretase, producing a truncated soluble fragment called sAPP β , which lacks the protective effects of sAPP α and may increase neuronal vulnerability, together with a membrane-associated fragment called β CTF [43-45]. The subsequent cleavage of β CTF by γ -secretase releases the A β peptide into the extracellular space. This peptide exists in several isoforms, the most common being A β 40, which is more soluble and less pathogenic, and A β 42, which is less abundant but more hydrophobic, unstable, and highly prone to aggregation.

2.1.3. Amyloid- β plaques formation and diffusion

The transition of the A β peptide from its monomeric form to the formation of pathological deposits follows a progressive sequence. Monomers first associate into soluble oligomers, which are considered the most neurotoxic entities because they directly disrupt synaptic transmission and neuronal plasticity [46-48]. These oligomers then evolve into protofibrils, unstable structures that reorganize into insoluble amyloid fibrils [49]. The accumulation of these fibrils in the extracellular space gradually leads to the formation of senile plaques, which are characteristic neuropathological markers of AD (**Figure 2**).

According to the classification proposed in 2002 by Thal and colleagues [50], five stages of amyloid deposition can be distinguished. In phase I, deposits appear in neocortical regions including the frontal, parietal, temporal, and occipital lobes. In phase II, they extend to the hippocampus, entorhinal and insular cortices, and the amygdala, among other structures. In phase III, deposits reach the basal ganglia, including the caudate nucleus, putamen, thalamus, and hypothalamus. In phase IV, they spread to the brainstem nuclei, and in phase V, they are finally detected in the cerebellum (**Figure 3**).

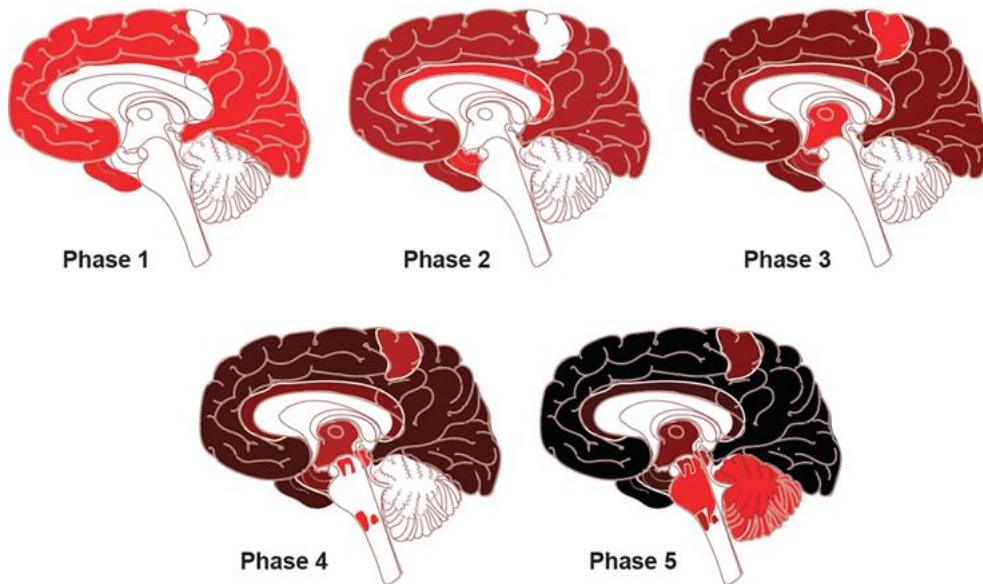


Figure 3. Thal staging of amyloid plaques. . Amyloid deposits progress from neocortical regions (phase I) to limbic areas (phase II), subcortical structures (phase III), brainstem nuclei (phase IV), and finally the cerebellum (phase V).

2.2. Tau pathology

2.2.1. Physiological role of the tau protein

The tau protein is encoded by a single gene, the Microtubule-Associated Protein Tau (*MAPT*), located on chromosome 17 and composed of 16 exons [51]. Initially described as neuron-specific, the expression of the *MAPT* gene has since been identified in various non-neuronal cell types [52-54]. In the central nervous system, alternative splicing of exons 2, 3, and 10 leads to the formation of six major tau isoforms [55]. These isoforms differ by the absence (0N), or the presence of one (1N) or two (2N) inserts of 29 amino acids in the N-terminal region (NTR) encoded by exons 2 and 3. The isoforms also differ by the number of repeated sequences within the microtubule-binding domain: three repeats (3R) when exon 10 is excluded, or four repeats (4R) when it is included [56-58] (**Figure 4A**).

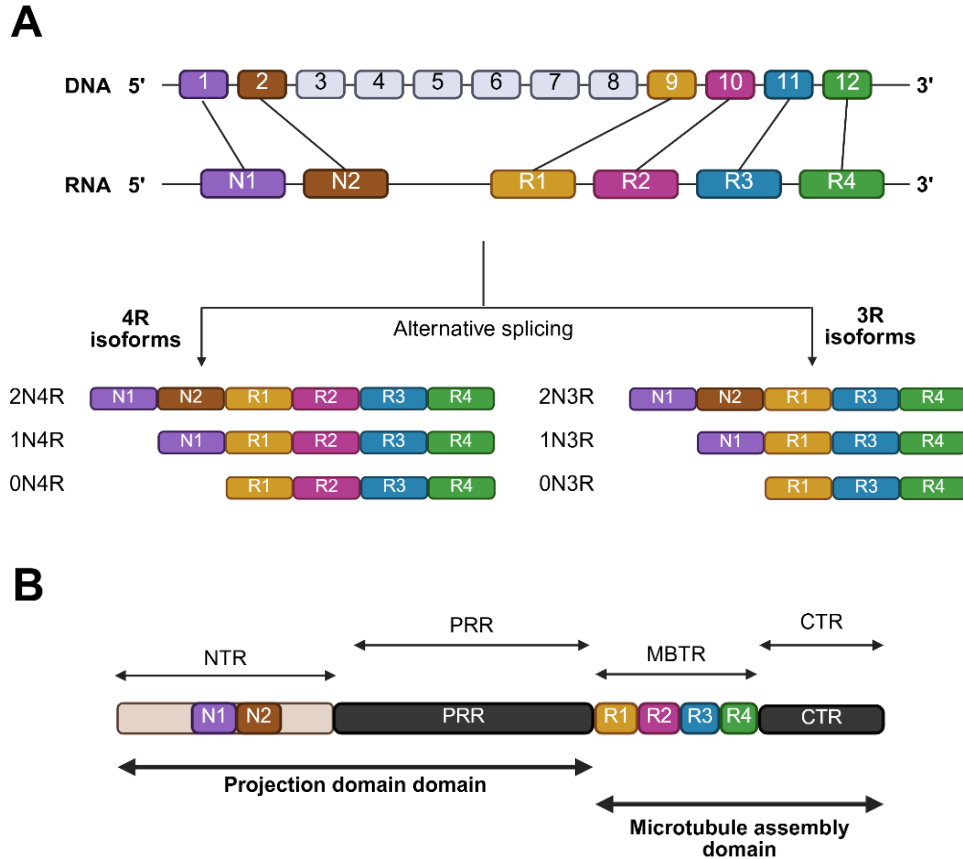


Figure 4. Tau isoforms through alternative splicing simplified. (A) Alternative splicing of the MAPT gene generates six major tau isoforms through the inclusion or exclusion of exons 2, 3, and 10. These isoforms differ by the number of N-terminal inserts (0N, 1N, or 2N) and by the presence of three (3R) or four (4R) microtubule-binding repeats. **(B)** These structural differences define the organization of tau into distinct domains, including the N-terminal region (NTR), the proline-rich region (PRR), the microtubule-binding region (MTBR), and the C-terminal region (CTR). Generated by biorender.

The six isoforms are therefore divided into two groups: the 3R forms (0N3R, 1N3R, 2N3R) and the 4R forms (0N4R, 1N4R, 2N4R) [59]. Under physiological conditions, the 3R and 4R isoforms are produced in similar amounts, keeping a 1:1 ratio [60]. The 4R forms have one extra binding sequence, which allows them to bind to microtubules more strongly and provide better cytoskeletal stability. The 3R forms

are more flexible and are mainly involved in neuronal growth and neurite extension [61].

Tau is an important protein that maintains the structure and stability of the neuronal cytoskeleton. By binding to microtubules, it controls their assembly and dynamics, which is essential for neuronal structure and plasticity. In addition to this structural role, tau interacts with many proteins and takes part in cell signaling, showing its key function in neuronal balance. Tau also regulates axonal transport by slowing kinesin movement and microtubule binding, and by affecting dynein activity to support retrograde transport [62-67].

Tau is a soluble and unstructured protein that contains many charged and hydrophilic residues. When it binds to microtubules, it becomes more ordered, showing that microtubules help shape its structure.

Structurally, tau is divided into four main regions with specific biochemical and functional properties. The amino-terminal region (NTR), encoded by exons 2 and 3, contains the 1N and 2N inserts and is rich in aspartic and glutamic acids [68]. It can interact with several kinases involved in tau phosphorylation. The proline-rich region (PRR) is also basic and helps in cell signaling by binding to various partners such as growth factors, DNA, and RNA. These two amino-terminal regions, both negatively charged, form the projection domain of tau [69, 70]. Because of their electrostatic repulsion from microtubules, this terminal region stays free and can interact with other cytoplasmic proteins [57, 71].

The microtubule-binding region (MTBR) is positively charged and is responsible for tau binding to microtubules through electrostatic interactions. It includes the 3R or 4R repeat sequences, which are essential for both binding and tubulin polymerization, playing a key role in microtubule stability. The carboxy-terminal region (CTR), which also has many phosphorylation sites, helps control tau activity and may interact with other cytoplasmic elements. Together, the MTBR and CTR form the microtubule assembly domain [72] (**Figure 4B-5**).

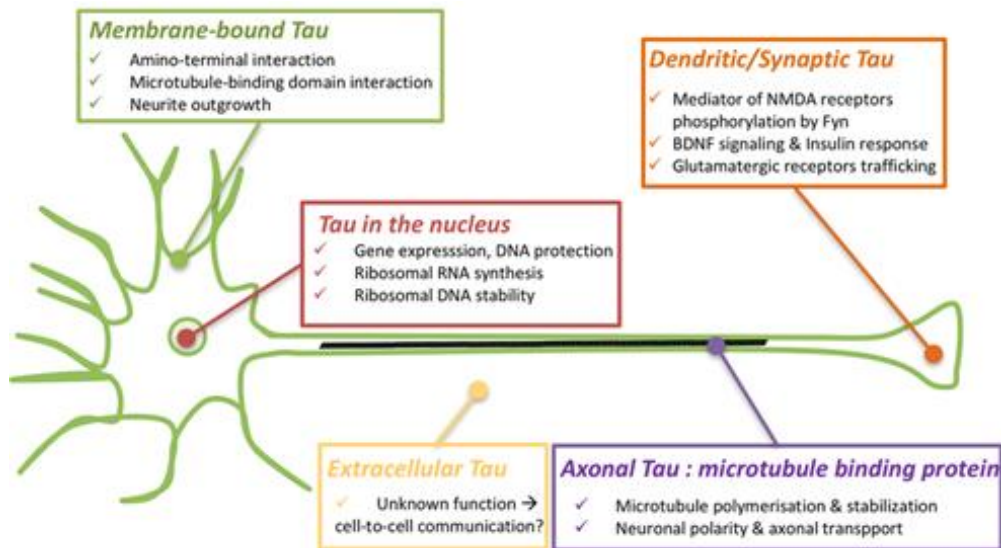


Figure 5. Physiological role of Tau protein in neuronal cell. Tau regulates microtubule assembly and stability, supporting neuronal structure and cytoskeletal organization. In addition, tau interacts with multiple proteins involved in cell signaling and modulates axonal transport by regulating motor protein activity.

2.2.2. Post-translational modifications of the tau protein

Under physiological conditions, several post-translational modifications (PTMs) regulate the functions of tau, including phosphorylation, ubiquitination, and acetylation [74, 75]. Ubiquitination, which mainly targets lysine residues, directs tau toward degradation by the proteasome. Acetylation of the same residues can block this process by competing with ubiquitin, thus prolonging the protein's half-life. Among these PTMs, phosphorylation is the most extensively studied, as it plays a central role in both normal and pathological regulation of tau [70, 76].

Approximately 20 percent of the tau sequence corresponds to potential phosphorylation sites, located on serine, threonine, and tyrosine residues [77]. Under normal conditions, the reversible addition of phosphate groups introduces extra negative charges that modify tau–microtubule interactions. Because microtubules are also

negatively charged, this increase in electrostatic repulsion reduces tau binding affinity, allowing dynamic regulation of microtubule stability and plasticity [74]. This flexibility is essential for proper cytoskeletal reorganization.

Tau phosphorylation is a reversible process tightly controlled by the coordinated action of kinases and phosphatases. Among the kinases, GSK3 β and Cdk5 are the main regulators of tau phosphorylation [40, 78-82]. Other kinases, such as mitogen-activated protein kinases (ERK, JNK, and p38), as well as protein kinases A and C (PKA and PKC), can also modify tau depending on the cellular context. Conversely, dephosphorylation is mainly carried out by protein phosphatase 2A (PP2A), which accounts for about 70% of total tau dephosphorylation activity in the brain [83-85]. Other phosphatases, such as PP1 and PP2B, also contribute to this process [86, 87].

The balance between the activities of these kinases and phosphatases determines the phosphorylation state of tau, maintaining its physiological function and preventing pathological deregulation.

2.2.3. Neurofibrillary tangles formation

In a pathological context, the dysregulation of tau phosphorylation observed in patients corresponds to two distinct phenomena. The hyperphosphorylation, characterized by an overall increase in the number of phosphorylated sites per tau molecule, and abnormal phosphorylation, which affects specific residues that are normally unphosphorylated under physiological conditions. These residues are mainly located within the PRR and the MTBR both essential for tau-microtubule interactions (**Figure 6A**).

Hyperphosphorylation causes extensive detachment of tau from microtubules, reducing their stability and leading to a gradual disorganization of the neuronal cytoskeleton. As a result, microtubules become unstable, disrupting axonal transport of vesicles, mitochondria, and other key elements required for cellular homeostasis. This process is accompanied by an abnormal

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delocalization of tau, which migrates from its usual axonal compartment to the soma and dendrites [60, 88, 89]. Such redistribution contributes to impaired synaptic signaling and weakened neuronal networks.

In parallel, hyperphosphorylated tau proteins undergo conformational changes that increase their tendency to self-associate. Initially, they assemble into unstable dimers, which then form soluble oligomers, now recognized as particularly toxic species because they directly disrupt synaptic transmission and intracellular transport [90, 91]. These oligomers further aggregate into protofibrils, and eventually into insoluble fibrils composed of highly ordered β -sheet-rich structures [92-95]. The progressive accumulation of these fibrils results in the formation of NFTs, abnormal insoluble intracellular inclusions that accumulate within the soma of neurons [33, 96] (**Figure 6B**).

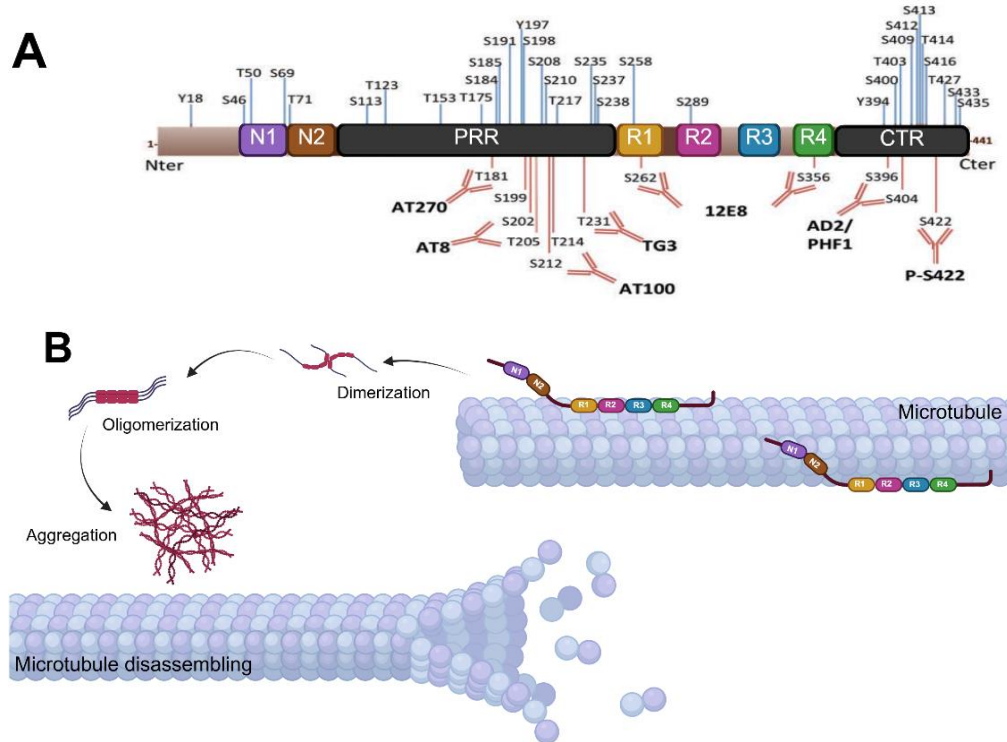


Figure 6. Hyperphosphorylation of tau protein. (A) In pathological conditions, tau undergoes abnormal and hyperphosphorylation, mainly affecting residues in the proline-rich region and the microtubule-binding region. (B) Hyperphosphorylated tau undergoes conformational changes that promote self-assembly into dimers and soluble oligomers, followed by the formation of protofibrils and insoluble fibrils. The accumulation of these fibrillar structures leads to the formation of neurofibrillary tangles within neurons. Generated by biorender.

These NFTs are not only key histopathological markers of AD but also active contributors to neuronal degeneration. Their presence severely disrupts the cytoskeletal structure, impairs axonal transport, and leads to synaptic loss. In addition, the accumulation of pathological tau activates pro-apoptotic pathways and promotes the trans-synaptic spread of toxic tau species, driving the gradual propagation of pathology throughout the brain.

2.2.4. Tau pathology spatio-temporal propagation

A major feature of tau pathology in AD is its ability to progressively spread throughout the brain. Two main hypotheses have been proposed to explain this phenomenon.

The first is the cellular vulnerability hypothesis, which suggests that some brain regions are naturally more sensitive to pathological changes. Local factors such as biochemical composition, energy metabolism, synaptic density, and the expression of kinases and phosphatases may explain why areas like the hippocampus and entorhinal cortex (EC) are affected early, before the pathology spreads to associative and neocortical regions. This first hypothesis made sense as it explained why the pathology developed in different brain regions at different times, following a pattern in which the most vulnerable areas were affected first, without involving any physical propagation [97-99].

The second hypothesis is the intracellular propagation of tau pathology along connected neuronal circuits, through a mechanism described as “prion-like” [100-102]. This term refers to the ability of a misfolded protein to induce a similar abnormal shape in normal proteins and to move from one cell to another, similar to what happens with prion diseases. This mechanism involves two main phases: a template (or seeding) phase and a propagation phase. During the template phase, hyperphosphorylated tau adopts an abnormal β -sheet-rich structure that acts as a pathological seed. This misfolded tau recruits normal soluble tau and induces its conversion into abnormal oligomeric and fibrillar forms. The newly misfolded tau proteins can then serve as additional seeds, amplifying the pathological process through a self-sustaining chain reaction. During the propagation phase, tau spread occurs through multiple pathways. Pathological tau can be actively secreted by neurons via extracellular vesicles such as exosomes or released into the extracellular space through non-vesicular mechanisms, including direct translocation across the plasma membrane or passive leakage from damaged cells [100, 102, 110].

Neighboring neurons can then internalize tau accumulation through receptor-mediated endocytosis. A direct cell-to-cell transfer can also occur through specialized structures called tunneling nanotubes, which create cytoplasmic continuity between neurons [111]. More recently, glial cells have been shown to participate in tau spreading: astrocytes and microglia can internalize pathological tau and subsequently release it, further promoting its dissemination and adding another level to intercellular propagation [112, 113] (**Figure 7A**).

Multiple experimental findings support this prion-like hypothesis [103, 104]. In animal models, intracerebral injections of extracts containing pathological tau have shown that tau can induce the aggregation of endogenous tau and progressively spread to anatomically connected brain regions [105-107]. In humans, tau PET imaging studies using specific radiotracers have confirmed a progression that closely follows neuronal networks, consistent with the neuropathological staging described by Braak [108, 109]. These observations provide strong evidence for the active cell-to-cell propagation of pathological tau.

Together, these mechanisms explain the characteristic spatiotemporal diffusion of tau pathology, systematically described by Braak through their neuropathological staging model.

2.2.5. Braak staging as the hierarchical progression of tau pathology

This progression differs from that of amyloid deposits, which appear in the neocortex before reaching deeper brain structures. In contrast, NFTs follow a distinct and hierarchical trajectory that begins in specific vulnerable regions and then spreads along connected neuronal circuits [114, 115] (**Figure 7B**).

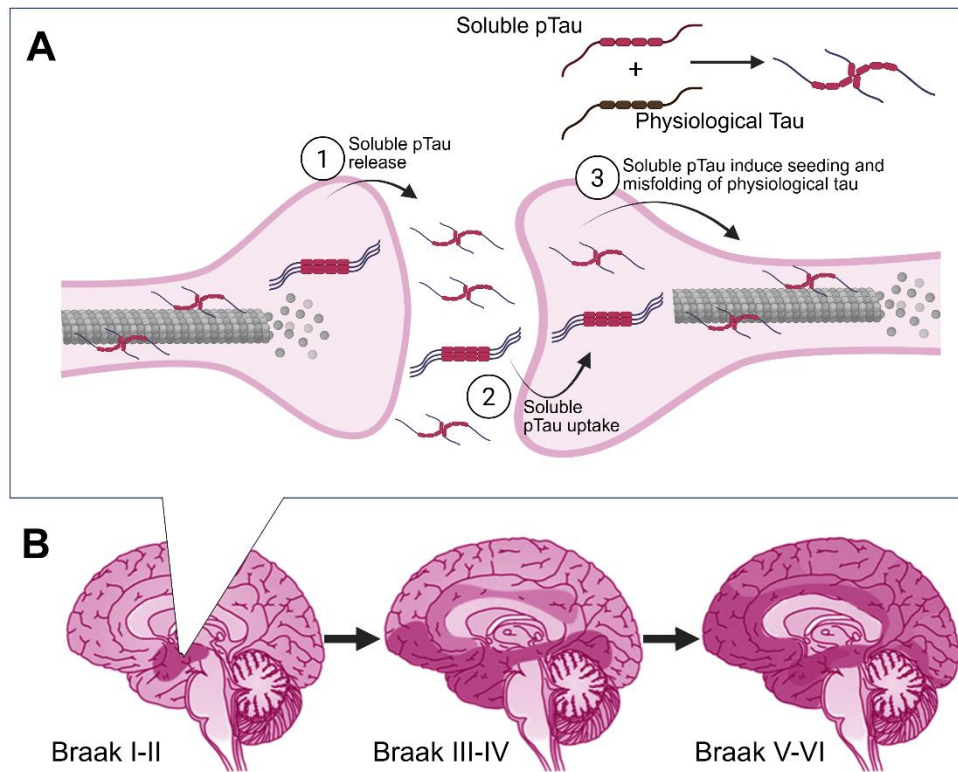


Figure 7. Prion-like propagation of tau pathology and Braak stages. (A) Prion-like propagation of tau pathology between connected neurons. Pathological soluble tau is released from an affected neuron, taken up by a neighboring neuron, and induces misfolding and aggregation of physiological tau through a seeding mechanism. **(B)** Progression of tau pathology in the brain according to Braak stages, from early involvement of medial temporal regions (Braak I–II), to limbic regions (Braak III–IV), and finally widespread neocortical areas (Braak V–VI). Generated by biorender.

In stage I, the first tau-related alterations are observed in the transentorhinal cortex, located at the interface between the hippocampus and the neocortex, as well as in the locus coeruleus, a brainstem structure involved in noradrenergic modulation. These regions thus represent the initial foci of NFTs formation.

In stage II, the pathology extends to the EC, which plays a key role in episodic memory and the multimodal integration of sensory

information. At this stage, very subtle cognitive symptoms may emerge, although most patients remain clinically asymptomatic.

Stages III and IV correspond to the spread of pathology to limbic structures, mainly the hippocampus and amygdala, two regions essential for memory, learning, and emotional regulation. At the same time, lesions gradually invade the associative cortex, particularly the unimodal and polymodal areas, which are responsible for integrating sensory inputs and coordinating higher cognitive functions. This extension accounts for the appearance of more pronounced memory deficits and the first behavioral changes.

Finally, in stages V and VI, NFTs become widespread throughout the neocortex, and ultimately, in the terminal phases, involving the primary sensory and motor cortices. At this stage, the pathology is diffuse and extensive, typically corresponding to the clinical manifestation of severe dementia, with significant loss of functional autonomy and global cognitive impairment.

Thus, Braak staging highlights a structured progression of tau pathology, following a gradient from the transentorhinal and limbic regions toward the neocortex [116-118]. It reflects the strong correlation between NFTs burden and clinical severity, in contrast to the distribution of amyloid deposits, whose presence does not show such a direct relationship with cognitive decline. This has led to the recognition of tau pathology as a major determinant of Alzheimer's disease symptoms, and Braak staging remains a key reference for the neuropathological assessment of disease progression.

3. Overview of the diagnostic approaches and treatments

AD lesions signatures, together with persistent neuroinflammation, progressively cause neuronal loss, which results in visible brain atrophy in the affected regions [70, 119, 120]. Neuronal death is accompanied by a reduction and alteration of synaptic connections, disrupting the brain's communication networks [70, 121]. It is now

widely established that the first lesions develop silently for nearly two decades before the onset of clinical symptoms.

Given this long preclinical phase, the diagnosis of AD relies on a combination of complementary approaches. For many years, *postmortem* neuropathological examination remained the gold standard for confirming the characteristic lesions. Today, clinical diagnosis mainly depends on the evaluation of cognitive and functional symptoms, supported by neuropsychological testing and the use of biomarkers. However, despite these advances, early diagnosis remains difficult, as the disease can be definitively confirmed only after clinical symptoms have appeared.

3.1. Postmortem neuropathological analyses

Although AD can be diagnosed based on clinical symptoms, a definitive confirmation requires postmortem examination [9]. Neuropathologists therefore analyze brain tissue to document the spatiotemporal progression of pathological deposits and to develop staging models such as Braak's for NFTs and Thal's for amyloid plaques [33, 122]. Immunohistochemistry plays a key role in these analyses, using specific antibodies against tau or A β that recognize different epitopes and reveal distinct stages or conformations of pathological accumulation.

Regarding tau protein, several antibodies have become reference tools [123]. Among them, AT270 is widely used as a biomarker for AD, as it detects tau phosphorylated at threonine 181, in tissue analysis and in CSF assays. The AT8 antibody, which recognizes tau phosphorylated at serine 202 and threonine 205, is now considered the most sensitive for detecting early tau pathology [124]. It allows the identification of early pre-tangle inclusions, when hyperphosphorylated tau begins to accumulate in the somato-dendritic compartment. Other antibodies, such as PHF-1 (targeting phosphorylation at serine 396/404) or AT100 (serine 212/threonine 214), preferentially label more advanced fibrillar inclusions, reflecting a later stage of tau pathology (Strang et al 2017). Finally,

conformational antibodies like MC-1, which recognize a specific abnormal structure of tau, allow visualization of early conformational changes independently of the phosphorylation status [125, 126] (**Figure 6A**).

In addition to antibodies, neuropathologists also use classical histological techniques, notably silver stains (Gallyas, or Bodian) [127-130], which permit sensitive detection of amyloid fibrils and tau inclusions. When combined with immunohistochemistry, these techniques provide accurate identification of the nature and developmental stage of pathological deposits.

Similarly, for amyloid plaques, the 4G8 and 6E10 antibodies, directed against specific sequences of the A β peptide, are commonly used to distinguish diffuse deposits from mature amyloid plaques [131, 132]. The combination of specific immunolabeling and histological staining remains the essential basis for the postmortem neuropathological diagnosis of AD.

3.2. Clinical diagnosis

The clinical diagnosis and management of AD rely on a multidisciplinary approach, involving the participation of several professionals. In Belgium, the diagnosis of AD is based on a multimodal approach combining cognitive evaluation via neuropsychological tests and the identification of disease-specific biomarkers [133] (**Figure 8A**).

3.2.1. Neuropsychological assessment

The first-line examination often remains the Mini-Mental State Examination (MMSE), widely used for its simplicity [134]. This test provides a global score of cognitive functioning, but its limitations are well known: it lacks sensitivity in the early stages of the disease, and its results are strongly influenced by the patient's educational level. For this reason, when cognitive impairment is suspected, a complete neuropsychological assessment is systematically preferred in specialized memory centers [134].

In Belgium, this neuropsychological battery is a crucial step. It aims to evaluate different cognitive functions in a detailed and systematic manner. Verbal episodic memory is assessed with sensitive tests that help distinguish retrieval difficulties caused by attention deficits from genuine storage impairments, which are characteristic of AD. Tests of visual and working memory complement this evaluation, as do assessments of language, executive functions, and attention. Integrating the results from these tests provides a detailed cognitive profile, which is essential not only to confirm the suspicion of AD but also to differentiate it from other neurodegenerative dementias (NDD) [135-142].

3.2.2. Biomarkers-based tools

When cognitive assessment suggests AD, the use of measurable and imaging biomarkers is recommended to confirm the diagnosis (**Figure 8A**). The analysis of cerebrospinal fluid (CSF), obtained through lumbar puncture, is currently considered the reference method. Three main biomarkers are measured: the decrease in amyloid- β 42 (A β 42), or more specifically in the A β 42/A β 40 ratio, reflecting cerebral amyloid deposition [143], the increase in total tau, indicative of axonal degeneration; and the elevation of phosphorylated tau (p-Tau181, or in some protocols p-Tau217 or p-Tau231), which more specifically reflects tau pathology and the formation of NFTs. This combined profile (low A β 42, high t-Tau and p-Tau) is considered characteristic of AD and provides very good diagnostic sensitivity and specificity.

In addition, molecular imaging techniques provide another major tool for visualizing pathological deposits *in vivo* [144-146]. Amyloid PET uses in clinical practice, fluorinated compounds such as [^{18}F]florbetapir, which bind to fibrillar amyloid plaques. This examination allows confirmation of the presence and distribution of amyloid deposits, often several years before the onset of clinical symptoms. Tau PET, a more recent approach, uses specific tracers such as [^{18}F]flortaucipir or [^{18}F]MK-6240. Unlike amyloid PET, tau PET is not yet used routinely in clinical settings, but it shows a strong

correlation with clinical severity and symptom topography, as the distribution of tau deposits follows the neuropathological progression described by Braak.

In parallel, magnetic resonance imaging (MRI) is an essential diagnostic tool. Structural MRI is the most used technique in clinical practice. It allows the detection of brain atrophy, particularly in the hippocampus, EC, and medial temporal lobe structures. These regions are affected early in the disease, and their atrophy provides strong diagnostic evidence, especially when it is asymmetric or greater than expected for normal aging. As the disease advances, atrophy extends to the limbic and associative cortices, reflecting the progressive spread of tau pathology described by Braak. Structural MRI is also essential for excluding other causes of cognitive decline, such as vascular lesions or tumors, making it a standard examination in any dementia workup [147].

Finally, fluorodeoxyglucose positron emission tomography (FDG-PET) serves as another biomarker of neurodegeneration (ND) [148]. This technique measures cerebral glucose metabolism, an indirect marker of neuronal activity. In AD, FDG-PET reveals a characteristic hypometabolism pattern that first affects the temporo-parietal regions, the posterior cingulate cortex, and the precuneus, before spreading to other associative areas. FDG-PET is therefore a sensitive indicator of neuronal dysfunction and is particularly useful for distinguishing AD from other dementias, which show different metabolic patterns.

3.3. Limitations of early diagnosis

The evolution of knowledge on biomarkers has revealed a characteristic temporal sequence in AD (**Figure 8B**). The first changes involve the amyloid cascade, reflected by a decrease in the A β 42/A β 40 ratio in the cerebrospinal fluid (CSF). This reduction occurs because the A β 42 peptide, being more hydrophobic, tends to aggregate within the brain tissue, thereby reducing its measurable concentration in the CSF. This process, directly related to the initiation of amyloid deposition, can be detected even before fibrillar plaques

become abundant enough to be visualized by amyloid PET. It defines the preclinical stage, during which a patient may already be amyloid-positive or even tau-positive while remaining clinically asymptomatic.

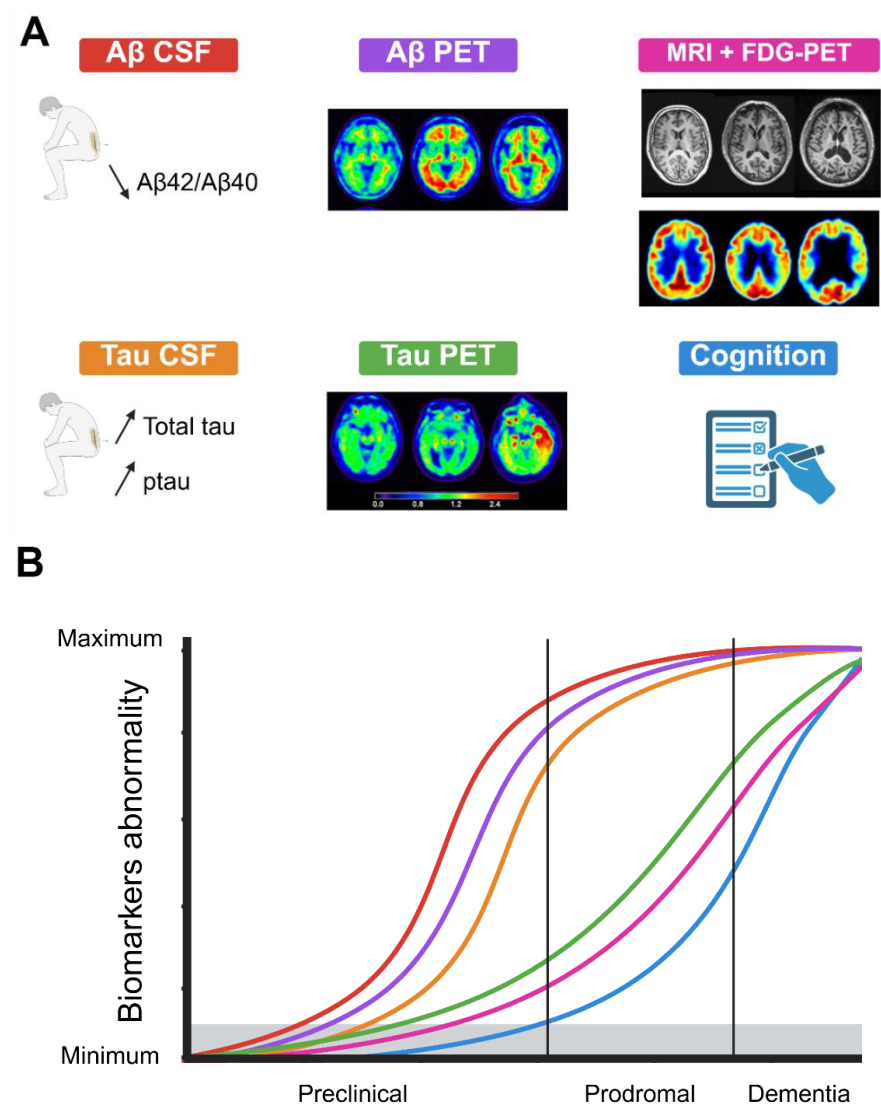


Figure 8. Alzheimer's Disease diagnostic tools and evolution. (A) Amyloid pathology is measured using cerebrospinal fluid (CSF) A β 42/A β 40 ratio and amyloid PET imaging. Tau pathology is assessed using CSF total tau and phosphorylated tau (p-tau), as well as tau PET imaging. Neurodegeneration is evaluated using MRI and FDG-PET, while cognitive performance is assessed using neuropsychological tests. (B) Temporal evolution of Alzheimer's disease biomarkers across disease stages. Amyloid biomarkers show early

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changes during the preclinical phase, followed by tau biomarkers during the prodromal phase. Markers of neurodegeneration and cognitive decline increase later and reach their maximum during the dementia stage. Generated by biorender.

These abnormalities are followed by an increase in phosphorylated tau protein levels in the CSF and by tau deposition detectable through PET imaging, reflecting the formation of NFTs. As long as these biological alterations are not accompanied by clinical manifestations, they are still considered part of the preclinical stage. The transition to the prodromal stage occurs when mild cognitive impairment (MCI) becomes detectable through neuropsychological evaluations but does not yet significantly affect functional autonomy.

Finally, markers of ND, such as hippocampal atrophy visible on structural MRI or cortical hypometabolism detected by FDG-PET, indicate more advanced neuronal damage. When these processes exceed the brain's compensatory capacity and are associated with multiple cognitive deficits that interfere with daily life, the condition corresponds to the dementia stage.

This sequential progression, now integrated into the A/T/N framework, which classifies AD based on biomarkers of amyloid (A), tau (T), and neurodegeneration (N) rather than clinical symptoms [133, 149-151]. This model emphasizes that biological changes precede clinical expression by many years [7]. During this preclinical phase, cognitive performance remains preserved likely as a result of compensatory mechanisms. Thus, when symptoms become noticeable and allow for objective diagnosis, significant neuronal damage has already occurred.

Despite major advances in biomarker research, early diagnosis of Alzheimer's disease remains limited by the tools currently available. Clinically validated methods such as CSF analysis or amyloid PET face major constraints, including the invasiveness of lumbar puncture, high cost, and limited access to imaging facilities. These factors restrict their use in patients with mild symptoms or isolated memory

complaints, even though this is the stage when diagnosis would be most useful [9].

To overcome these limitations, the research is now focused on developing blood-based biomarkers. Very promising results have been obtained with plasma measurements of the A β 42/A β 40 ratio, phosphorylated tau isoforms (p-Tau181, p-Tau217, p-Tau231) [152-154]. Their use could allow non-invasive, accessible, and cost-effective detection of the disease. Although still under validation, these biomarkers pave the way for a true diagnostic revolution, offering the potential for large-scale early screening, either as a complement or eventually a substitute for currently invasive and expensive examinations.

3.4. Alzheimer's Disease treatments

The management of patients diagnosed with AD should start as early as possible. Patients are monitored through regular clinical follow-ups and receive treatment to slow down the neurodegenerative process and reduce symptoms. Two main classes of drugs are currently approved for AD: acetylcholinesterase inhibitors (AChIs) and memantine, an N-methyl-D-aspartate (NMDA) glutamate receptor antagonist [155].

Cholinesterase inhibitors, such as donepezil, are prescribed for mild to moderate stages of the disease to improve cognitive functions. These inhibitors prevent the breakdown of acetylcholine (ACh), a neurotransmitter essential for synaptic function, within the synaptic cleft. As the disease progresses, neuronal networks are destroyed, and the number of post-synaptic nicotinic and muscarinic receptors that respond to ACh decreases. Therefore, by blocking acetylcholine degradation, these inhibitors increase the availability of ACh in the synaptic cleft, enhancing cholinergic transmission and supporting cognitive stimulation [155].

Memantine is the only approved NMDA receptor antagonist for AD. It is recommended for moderate to severe stages, when AChIs alone are

no longer effective. Glutamate normally acts on NMDA receptors to promote long-term potentiation, a key process for learning and memory. Under pathological conditions, excessive glutamate in the synaptic cleft overstimulates NMDA receptors, leading to neuronal damage through excitotoxicity. Memantine helps limit this overactivation, thereby restoring a more balanced glutamatergic transmission.

These current treatments are only symptomatic and have shown no curative effects, even at early stages of the disease. Many ongoing studies aim to develop new therapies that can modify the progression of Alzheimer's disease. The main therapeutic targets are the amyloid and tau pathologies, which are the two major lesions of the disease [155].

Anti-amyloid therapies focus on reducing the accumulation of amyloid-beta ($A\beta$) peptides that form senile plaques in the brain. Two main strategies are used: vaccines and monoclonal antibodies.

Vaccines aim to stimulate the patient's immune system to produce antibodies against $A\beta$, helping to clear existing plaques or prevent new ones from forming. Animal studies showed that immunization with $A\beta_{42}$ prevented or reduced amyloid deposits and improved memory functions [156, 157]. However, early clinical trials such as the AN1792 vaccine were stopped due to inflammatory side effects [155]. New vaccine generations using modified peptides are now under investigation and appear to be better tolerated.

Monoclonal antibodies directly target $A\beta$ and promote its clearance by immune mechanisms. Some antibodies, like *bapineuzumab* and *solanezumab*, showed limited clinical effects [158, 159]. More recently, *aducanumab* and *lecanemab* demonstrated a reduction of amyloid plaques and a modest slowing of cognitive decline, leading to FDA approval for early Alzheimer's disease [160].

Anti-tau therapies target the accumulation of hyperphosphorylated tau proteins forming neurofibrillary tangles. Vaccines and monoclonal

antibodies are designed to recognize and remove pathological tau or stop its spread between neurons [155, 161]. Although still in early clinical phases, these approaches are promising since tau pathology correlates more closely with cognitive decline than amyloid load.

Overall, anti-amyloid and anti-tau immunotherapies are among the most promising disease-modifying strategies. By targeting the two main molecular mechanisms of AD, they may one day lead to more effective and potentially preventive treatments [157, 162].

II. Link between Alzheimer's Disease and olfaction

As mentioned previously, from a histopathological perspective, NFTs described by Braak first appear in brain regions such as the transentorhinal cortex, followed by the entorhinal cortex and hippocampus. These areas are essential for memory and spatial navigation and maintain close anatomical connections with the olfactory structures. Functionally, olfactory dysfunction is observed in about 85-90% of patients with AD [103, 163]. However, this impairment is not specific to AD, as it is also reported in other NDDs, highlighting the role of olfaction as a common but early marker of ND.

1. Olfactory functions and associated anatomical structures

The olfactory system constitutes a complex sensory network composed of two main components: a peripheral part, responsible for detecting odorant molecules, and a central part, responsible for processing, integrating, and consciously perceiving olfactory information. Its hierarchical organization, from the olfactory epithelium to higher cortical areas, reflects the close interaction between olfaction and cognition (**Figure 9**).

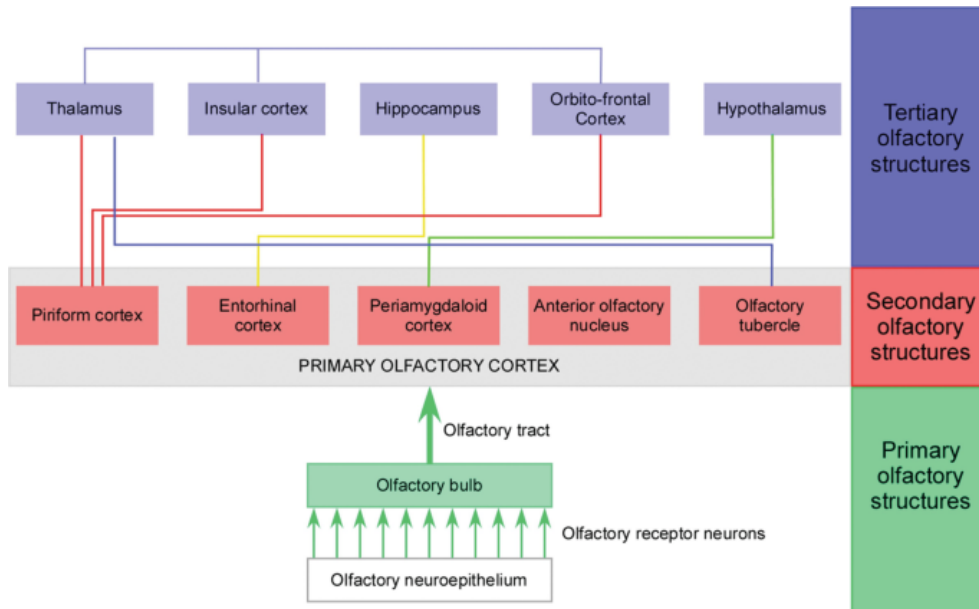


Figure 9. Olfactory system. Olfactory information is transmitted from olfactory receptor neurons in the olfactory neuroepithelium to the olfactory bulb, and then through the olfactory tract to primary olfactory cortical areas. These primary areas project to secondary and tertiary olfactory structures, supporting higher-order processing of olfactory signals.

The peripheral part of the olfactory system mainly corresponds to the olfactory neuroepithelium (ONE), located in the posterosuperior region of the nasal cavity, just below the cribriform plate of the ethmoid bone. The ONE is a pseudostratified epithelium composed of several cell types arranged in layers. The olfactory sensory neurons form the majority of these cells. They are bipolar neurons whose dendrites carry cilia immersed in mucus, where olfactory neurons detect odorant molecules [164]. These neurons are unique because they are continuously renewed, with an average lifespan of 4 to 8 weeks, giving the epithelium a remarkable capacity for adult neurogenesis [165]. The supporting cells (or sustentacular cells) have metabolic and structural functions. They help detoxify harmful substances and secrete odorant-binding proteins that solubilize hydrophobic molecules. The basal cells, located along the basal lamina, form a pool of stem cells that can generate both new sensory neurons and supporting cells [166]. Finally,

Bowman's glands, located in the connective tissue, secrete the mucus that covers the epithelial surface and allows odorant molecules to dissolve (**Figure 10B**).

Odor detection begins when these molecules bind to receptors on the cilia of olfactory neurons, generating an electrical signal that travels along their axons. The axons of olfactory neurons group together to form the olfactory nerve (cranial nerve I), which passes through the cribriform plate and reaches the olfactory bulb (OB) which is the first relay of the central olfactory system [167].

The OB, located on the ventral surface of the frontal lobe, is a highly organized structure composed of six layers. Within its glomerular layer, axons of sensory neurons converge and form synapses with the dendrites of mitral and tufted cells, the main projection neurons of the bulb. This synaptic organization allows initial processing and spatial coding of odors. A network of inhibitory interneurons (periglomerular and granule cells) further refines the signal, enhancing contrast and odor discrimination before transmission to higher centers (**Figure 10A**).

From the bulb, olfactory signals are relayed via the olfactory tract to several structures of the primary olfactory cortex (OC): the anterior olfactory nucleus (AON), the piriform cortex, the olfactory tubercle, the amygdala, and the EC [167, 168]. Each of these regions has a distinct functional role.

The AON constitutes a key relay and modulatory center within the olfactory pathway, positioned along the olfactory tract between the bulb and the more posterior cortical areas. Anatomically, the AON is subdivided into several regions including the bulbar portion (*pars bulbaris*), located within the OB [167-169]. Functionally, the AON plays a critical role in odor generalization, contrast enhancement, and context-dependent modulation of olfactory perception, acting as a dynamic integrative hub between sensory input and higher cortical processing.

Processed information is then transmitted to the primary olfactory cortex (OC), which includes the piriform cortex, the entorhinal cortex (EC), and the amygdala [167, 168]. Among the putative roles, the piriform cortex is implicated in odor identification and pattern recognition. The EC links olfactory input to the hippocampus, integrating odors with memory, while the amygdala gives odors emotional value [170].

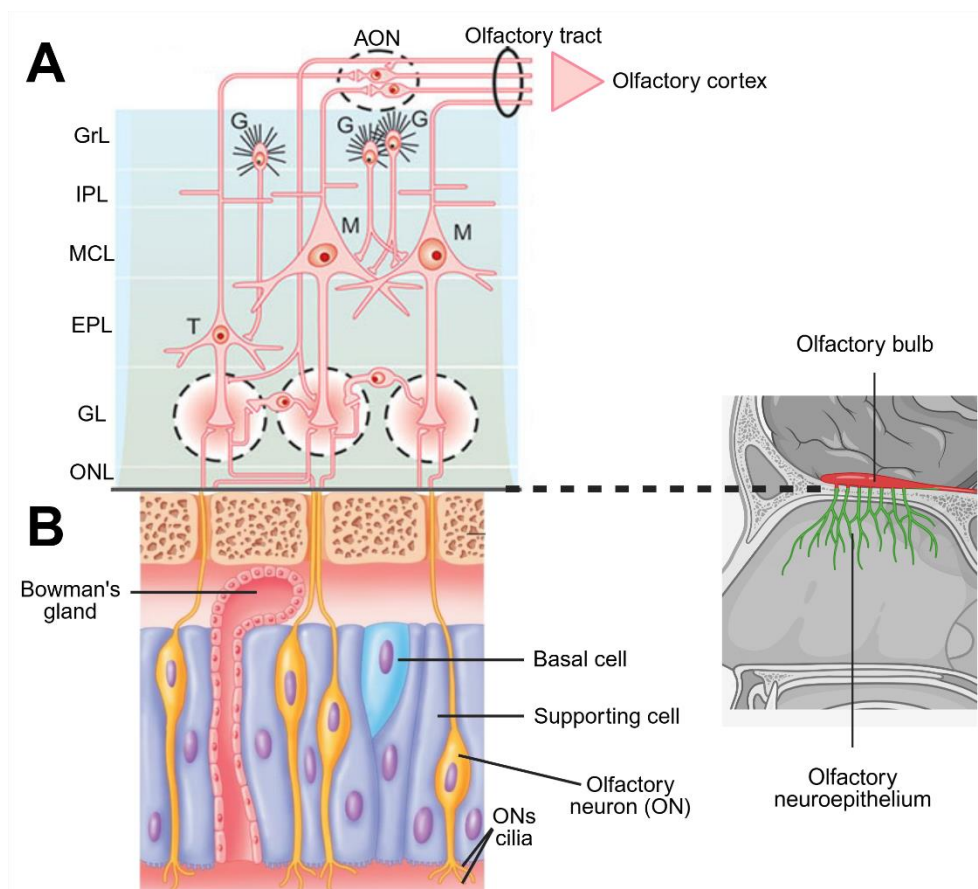


Figure 10. Cytoarchitecture of the human olfactory bulb and neuroepithelium. (A) Schematic organization of the olfactory bulb, showing its main cellular layers and neuronal circuits that relay olfactory information to the olfactory cortex via the olfactory tract. **(B)** Structure of the olfactory neuroepithelium, including olfactory sensory neurons, supporting cells, and basal cells, which together ensure odor detection and signal transduction. AON: anterior olfactory nucleus; EPL: external plexiform layer; GL: glomerular layer; GrL: granule cell layer; IPL: internal plexiform layer; MCL: mitral cell layer; M: mitral cell; T: tufted cell; ON: olfactory neuron; ONL: olfactory nerve layer. Generated by biorender.

Finally, olfactory information reaches tertiary cortical regions, such as the orbitofrontal cortex, insula, and anterior cingulate cortex, where conscious perception occurs [171]. These regions integrate sensory, cognitive, and emotional aspects, supporting higher-level functions such as odor naming and hedonic evaluation.

In addition to this ascending transmission, the olfactory system also operates through descending, or retrograde, pathways. These bidirectional connections ensure continuous communication between the OB and higher olfactory centers. The anterograde flow carries sensory information from the ONE to the OB and then to cortical regions, supporting odor detection, discrimination, and identification. In parallel, retrograde feedback from the AON, piriform cortex, and EC modulates the activity of the OB. This descending control adjusts the response level of olfactory neurons according to attention, context, and learning experiences [168, 169, 172]. Such reciprocal connectivity allows the olfactory system to integrate sensory input with memory and emotion, optimizing odor perception and behavioral responses (**Figure 11**).

Thus, the olfactory system is organized into a hierarchy of stages: molecular detection by the ONE, initial processing and filtering within the bulb, integration within limbic and cortical structures, and finally, conscious perception in the orbitofrontal cortex. This organization underlies the different olfactory functions measurable in humans such as detection threshold, discrimination, recognition, and identification of odors. Due to its cellular richness, the diversity of its relays, and its direct interconnection with memory and emotional regions, the olfactory system represents a unique model for understanding the mechanisms of olfactory dysfunctions observed in NDD such as AD.

2. Olfactory dysfunction as early indicator in Alzheimer's Disease

Several longitudinal studies have shown that individuals with olfactory deficits have a higher risk of developing cognitive impairment and,

later, AD. In initially healthy subjects, difficulty identifying odors predicts the transition to MCI and faster cognitive decline [173]. Population-based studies have confirmed that olfactory identification is a strong prognostic marker, sometimes even more accurate than certain memory tests for predicting decline and the onset of AD [174, 175].

From a structural point of view, MRI studies have shown a reduction in the volume of the OB and olfactory tract even in the early stages of AD. This atrophy is associated with medial temporal lobe shrinkage, suggesting that olfactory atrophy is part of the neurodegenerative continuum [176].

These findings are consistent with histopathological evidence showing that AD markers are present not only in central olfactory regions (OB, tract, piriform cortex, entorhinal and hippocampal areas) but also in peripheral olfactory tissues such as the ONE [177]. This distribution matches Braak's description of early transentorhinal and entorhinal involvement, which are strongly connected to olfactory pathways, supporting the idea that olfactory loss results from lesions affecting key regions of the olfactory system.

Taken together, these results indicate that olfaction should be considered a sensitive clinical marker of ND in AD.

3. Tau pathology propagation within the olfactory system

Tau accumulation in AD follows a stereotyped pattern that begins in central olfactory regions, such as the transentorhinal and entorhinal cortices, before extending to wider cortical and limbic networks. This topography implies that smell loss may primarily reflect early tau-driven ND rather than amyloid-related changes.

In clinical studies, poor odor identification has been associated with higher tau burden measured in CSF and with increased tau signal on PET imaging, even after accounting for amyloid status [178]. Olfactory scores also correlate with markers of glial activation and neuroinflammation, further supporting a link between olfactory

dysfunction and tau-related neuronal stress [178]. Longitudinal imaging studies reinforce this idea: reduced odor identification predicts later tau accumulation, especially in entorhinal and temporal regions that are part of the olfactory network [179].

In contrast, the relationship between olfactory deficits and amyloid load appears weaker and more inconsistent. Some studies report that better olfactory performance is associated with lower amyloid positivity and slower memory decline [180], but meta-analytic evidence indicates only a negligible connection between olfactory measures and amyloid deposition [181]. *Postmortem* analyses also show a predominance of tau over amyloid in olfactory structures such as the piriform and entorhinal cortices, suggesting that olfactory impairment reflects tau pathology more directly.

Given this association, it remains to be clarified how tau pathology involves the olfactory system, whether it spreads from central to peripheral regions or originates peripherally and progresses inward. Most evidence supports a central origin. According to Braak staging, tau pathology first appears in transentorhinal, entorhinal, and hippocampal regions, which are directly connected to the OB and olfactory cortex. This pattern suggests that tau spreads along central olfactory networks in a trans-synaptic, prion-like manner [179, 182].

However, other studies propose a peripheral entry hypothesis, in which the ONE, exposed to the external environment and capable of neurogenesis, could serve as an initial site for tau aggregation. From there, tau could propagate centripetally through the olfactory nerve and bulb toward central regions. Abnormal tau deposits have indeed been found in human olfactory mucosa and other peripheral tissues [183-185], and intranasal experimental models support this possible route of spread.

Current evidence therefore remains mixed (**Figure 11**). Some studies highlight the vulnerability of the OE and its potential as a peripheral biomarker source [186], whereas others emphasize the strong

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correspondence between olfactory dysfunction and central tau load, consistent with Braak's progression [179, 187].

Taken together, available data indicate that tau propagation within olfactory pathways is mainly centered on cortical and limbic regions, while peripheral olfactory tissues may reflect, rather than initiate, this process. Clarifying the direction and mechanisms of this propagation will help to better understand the role of olfactory dysfunction in AD and refine its use as an early clinical marker of tau pathology.

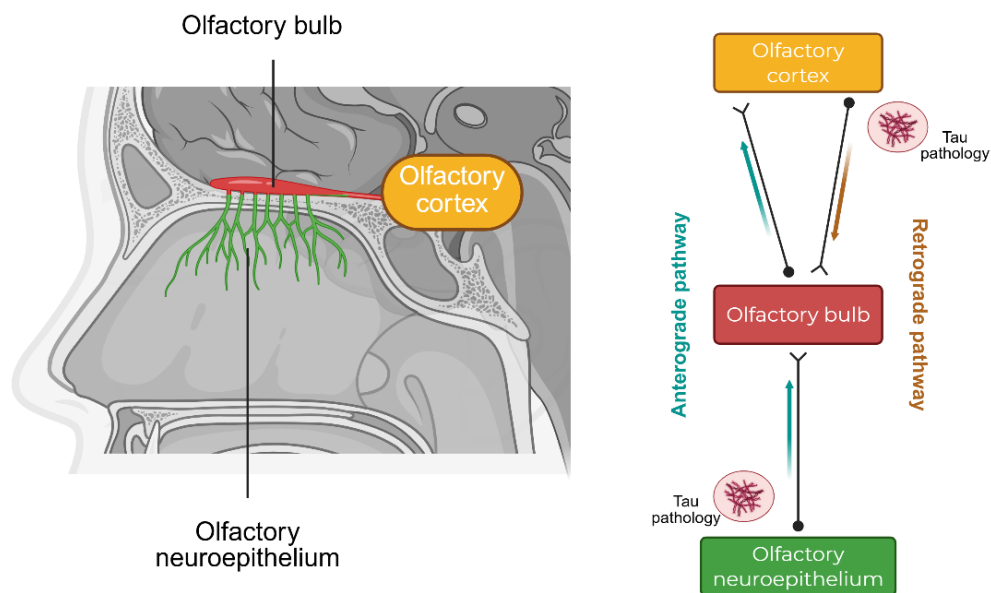


Figure 11. Hypothesis of tau propagation within the olfactory system. This schematic illustrates the hypothesis that tau pathology may propagate within the olfactory system through either an anterograde pathway, from the olfactory neuroepithelium (ONE) to the olfactory bulb and olfactory cortex, or a retrograde pathway, from the olfactory cortex back to the olfactory bulb. Generated by biorender.

CHAPTER 2

I. Biological aging and cellular senescence

1. Definition of aging

Aging is a natural biological process that leads to a gradual decline in the body's functional abilities. This decline results in reduced physiological performance, increased vulnerability to disease, and eventually death. According to López-Otín et al. [188], aging is not just random damage over time, but a regulated process influenced by molecular, cellular, and systemic factors that disturb the body's balance. Although aging looks different across species and tissues, a common set of mechanisms called the "hallmarks of aging" define its biological nature. These hallmarks share three conditions: they appear gradually with age; their worsening accelerates aging and reducing them can delay or improve age-related decline [188].

In humans, aging involves a gradual loss of function across multiple systems such as the heart, immune system, muscles, and brain. This process is driven by molecular damage, impair cellular repair, altered intercellular communication, and chronic inflammation. The difference between chronological and biological age, measured by physiological and molecular markers, shows that aging is dynamic and varies between individuals [188]. Aging results not only from damage accumulation but also from how the body responds to this damage through stress responses, metabolic changes, and compensatory mechanisms that eventually become harmful.

López-Otín and colleagues [188] identified twelve connected hallmarks of aging, divided into three groups (**Figure 12**). The primary hallmarks include genomic instability, telomere shortening, epigenetic changes, and loss of proteostasis. These represent the possible initial sources of molecular damage. The antagonistic hallmarks include deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence. These are adaptive responses that become harmful

when chronic. Finally, the integrative hallmarks include stem cell exhaustion, altered intercellular communication, and chronic inflammation. These reflect the consequences that drive tissue and organismal aging [188].

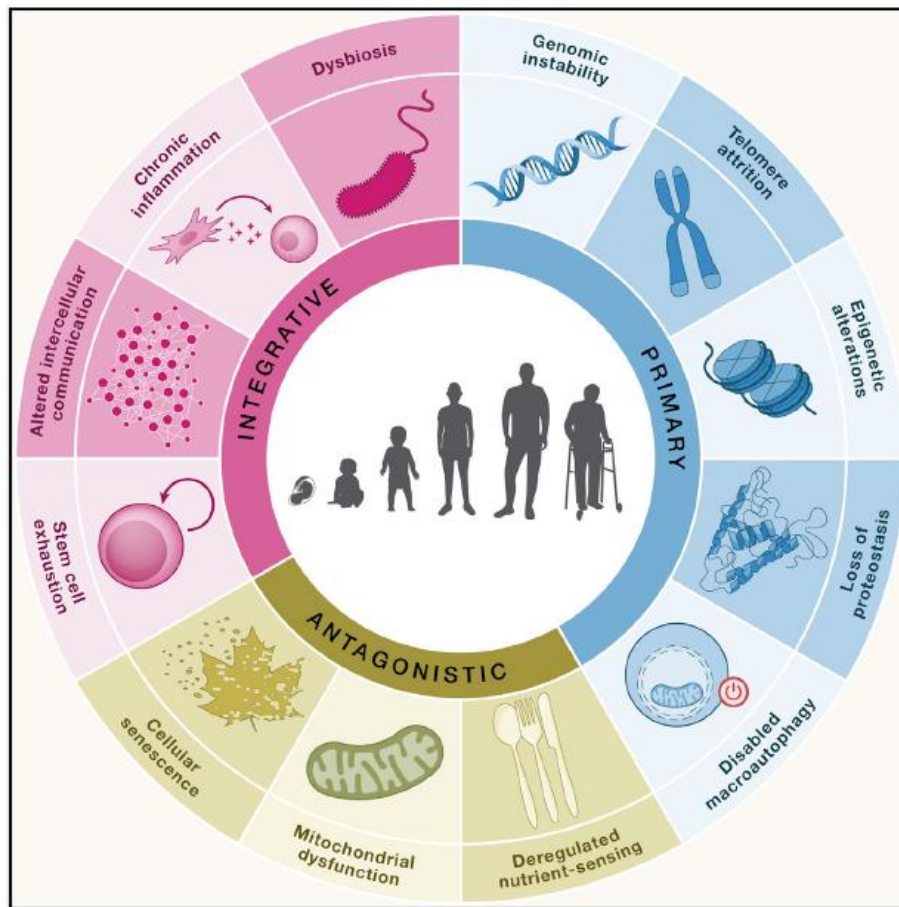


Figure 12. Hallmarks of aging. The figure summarizes the main biological hallmarks of aging, grouped into primary, antagonistic, and integrative processes which together drive functional decline during aging. From Lopez-Otin et al. 2023

Cellular senescence is a stable state in which cells permanently stop dividing in response to stress. Physiologically, senescence protects the organism by stopping the proliferation of damaged or precancerous cells [189, 190]. It also has roles in embryonic development and tissue repair by supporting remodeling and immune

signaling [191, 192]. However, if the immune system fails to clear senescent cells, they begin to accumulate in tissues over time. Their numbers can increase dramatically with age, reaching up to twenty times higher in older adults compared to younger individuals [188]. Their persistent presence promotes chronic inflammation, tissue damage, and stem cell exhaustion, contributing to age-related diseases such as neurodegeneration, atherosclerosis, diabetes, and cancer [188]. This dual role reflects that senescence helps protect the body when young but causes harm when it lasts too long with age.

2. The primary hallmarks of senescence

The mechanisms that drive cellular senescence are not yet fully understood and probably differ across cell types and tissues. The classification by López-Otín et al. [188] proposes the existence of five inducers of cellular senescence: telomere attrition, genomic instability, epigenetic alterations, loss of proteostasis, and impaired macroautophagy. In the context of this PhD thesis, we investigated telomere attrition and loss of proteostasis as possible drivers of cellular senescence in our biological samples.

2.1. Telomere attrition and replicative senescence

2.1.1. Replicative senescence *in vitro*

The phenomenon of replicative senescence was first described by Hayflick and Moorhead [193], who observed that normal human fibroblasts in culture stop dividing after a limited number of divisions, even when nutrients are sufficient. This “Hayflick limit” showed that normal cells have an internal replication boundary caused by an intrinsic program, not by cell death or lack of resources (**Figure 13**).

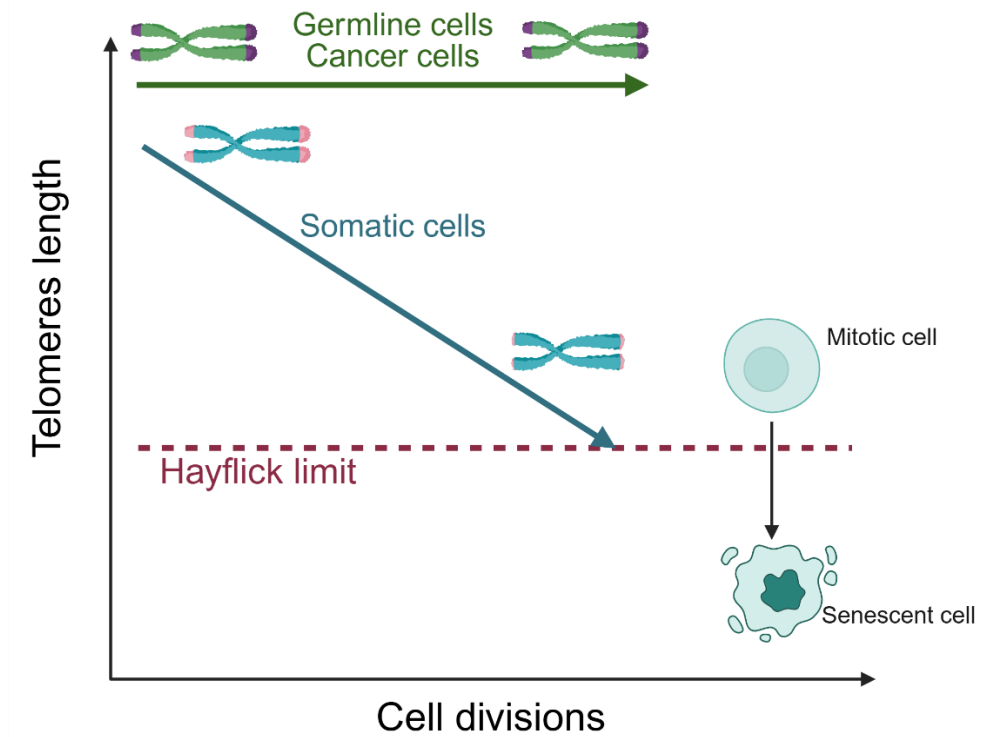


Figure 13. Hayflick limit to explain senescence. With each cell division, telomeres progressively shorten in somatic cells. When telomere length reaches a critical threshold, known as the Hayflick limit, cells permanently stop dividing and enter senescence. In contrast, germline and cancer cells maintain telomere length, allowing continued cell division. Generated by biorender.

Later studies revealed that this limit results from progressive telomere shortening, which activates a persistent DNA damage response (DDR) [194, 195]. Telomeres are DNA–protein structures located at the ends of chromosomes, composed of TTAGGG repeats bound by the shelterin complex (TRF1, TRF2, TIN2, TPP1, POT1, RAP1). This complex protects chromosome termini and regulates telomerase activity [196]. Because DNA polymerases cannot fully replicate chromosome ends, telomeres in normal somatic cells shorten with each cell division, a process accelerated by oxidative and replication stress [197]. When telomeres become too short, they are recognized as double-strand breaks. The MRE11-RAD50-NBS1 complex detects this damage and recruits the ATM and ATR kinases, which then

phosphorylate histone H2AX. The resulting γ -H2AX foci mark persistent damage sites known as telomere dysfunction–induced foci (TIF) that signal the initiation of senescence [188, 194, 195].

The stable growth arrest that defines cellular senescence is controlled by the p53/p21 and p16/pRB tumor suppressor pathways [190, 198]. After DDR activation, the kinases ATM and ATR stabilize TP53, inducing CDKN1A (p21) expression. p21 inhibits CDK2–cyclin complexes, causing hypophosphorylation of pRB and suppression of E2F-dependent genes needed for DNA replication. At the same time, CDKN2A (p16) blocks CDK4/CDK6, reinforcing pRB activation and maintaining G1 arrest. While p21-mediated arrest may be reversible, the involvement of p16 and chromatin remodeling makes senescence irreversible [199, 200]. In aging tissues, chronic activation of these pathways promotes stem cell exhaustion and tissue decline [188, 201].

2.1.2. Telomere erosion and TIF in organismal aging

While telomere erosion and TIF are well-established drivers of replicative senescence *in vitro*, their contribution to normal organismal aging remains debated [194].

What is clear however is that in patients suffering from genetic diseases known as telomeropathies or telomere biology disorders (TBDs), the accelerated telomere shortening triggers premature cellular senescence and early tissue failure [202]. These inherited diseases often manifest as bone marrow failure, pulmonary fibrosis or hepatic cirrhosis, mirroring key features of [203, 204]. The most frequent causes involve mutations in *TERT*, *TERC* or *RTEL1*, which affect distinct but complementary aspects of telomere biology. Mutations in *TERT* and *TERC* impair telomerase function, reducing its ability to compensate for telomere shortening [204].

Beyond these pathological conditions, studies have shown that telomere dysfunction can also occur during normal physiological aging *in vivo*. A study [205] demonstrated that TIF progressively accumulate with age in small airway epithelial cells of mice, even without

detectable telomere shortening. This finding suggests that telomere-associated DNA damage can arise independently of telomere length loss, possibly due to oxidative stress or inefficient repair at telomeric regions. Similarly, another study [206] identified TIF in human lung tissues from aged individuals, supporting the idea that low-level telomere damage is a natural feature of tissue aging. Together, these studies indicate that telomere dysfunction may be a physiological marker of aging in vivo, reflecting the cumulative impact of chronic stress and limited repair capacity rather than a consequence of critically short telomeres.

However, it remains uncertain whether the frequency of TIF consistently increases with age across tissues or directly contributes to functional decline. More work using samples from very old individuals and different organs is required to clarify the role of age-associated telomere dysfunction in normal tissue aging.

2.2. Lysosomal accumulation and senescence associated- β -galactosidase activity

One of the earliest and most widely used biomarkers of senescence is the increase in lysosomal content and enzymatic activity, detected as senescence-associated β -galactosidase (SA- β -gal) activity at pH 6.0 [207]. This marker reflects lysosomal expansion due to increased autophagy and synthesis of lysosomal enzymes. Lysosomal enlargement helps senescent cells survive by degrading damaged components, even though they can no longer divide. However, this adaptation also has negative effects: excessive lysosomal activity can lead to lipofuscin accumulation and raises reactive oxygen species (ROS) levels, contributing to the oxidative stress typical of aging cells [208]. Thus, increased lysosomal activity serves both as a key biomarker and as a contributor to maintain senescence.

In senescence, the accumulation of damaged proteins exceeds the cell's degradation capacity, disrupting proteostasis. As a result, mitophagy becomes impaired, leading to mitochondrial dysfunction and elevated production of mitochondrial ROS. This rise in oxidative

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stress further damages DNA and accelerates telomere shortening, creating a feedback loop that reinforces and stabilizes the senescent phenotype.

OBJECTIVES OF THE THESIS

The spread of tau pathology across cortical regions is well characterized by Braak staging. However, its propagation within the olfactory system remains poorly understood. Previous studies have reported the presence of tau pathology in the OB as early as Braak stage II, following initial involvement of central regions. In contrast, data concerning the ONE are scarce and inconsistent, mainly because most studies have examined late-stage cases, limiting insights into potential early involvement.

The olfactory system has recently gained attention as olfactory dysfunctions have been recognized as early indicators of AD, with a particularly strong association with tau pathology. These findings have led to the hypothesis that tau pathology could originate in the ONE which is composed of olfactory sensory neurons, and subsequently, spread toward central olfactory regions.

The main objective of this thesis was therefore to better map the progression of tau pathology within olfactory regions, including the OB and the neuroepithelium, in order to explore potential early intervention or diagnostic windows for AD. To achieve this, we combined post-mortem analyses of the human OB across different Braak stages with nasal brushing techniques providing ex vivo access to olfactory sensory neurons from patients along the AD continuum. In parallel, we performed olfactory function tests assessing multiple dimensions of smell perception that reflect the activity of different olfactory regions.

In a second part of this work, taking advantage of the nasal brushing technique, we focused on the mechanisms of aging, whose major hallmark is cellular senescence. Senescence is characterized by multiple molecular and cellular markers; however, the upstream triggers of this process remain poorly defined and appear to vary depending on the tissue studied. We therefore investigated cellular senescence within the olfactory epithelium, using nasal brushing as a minimally invasive method allowing rapid exfoliation and access to this tissue. We analyzed samples obtained from participants across a wide

age continuum, as well as from patients with telomeropathies, which represent a model of premature aging.

RESULTS

RESULTS-CHAPTER I

Olfactory decline tracks central-to-peripheral spread of tau pathology in
Alzheimer's disease

Preamble chapter I: The olfactory system is particularly intriguing because, although it is organized into both central and peripheral components, its peripheral neurons, present in the ONE share key properties with those of the CNS. Notably, olfactory neurons express the tau protein through alternative splicing, giving rise to isoforms that closely resemble those found in the CNS, rather than the “Big Tau” variant typically observed in the peripheral nervous system (PNS) (**Fig. 1'**). In the context of neurodegenerative diseases such as Alzheimer's disease, ONS offer several practical and biological advantages for studying disease mechanisms. One of the most significant is their accessibility, as olfactory neurons can be obtained through relatively non-invasive nasal swabbing, making them a valuable model for investigating pathological processes in living patients. In particular, regarding the spread of tau pathology within the human olfactory system.

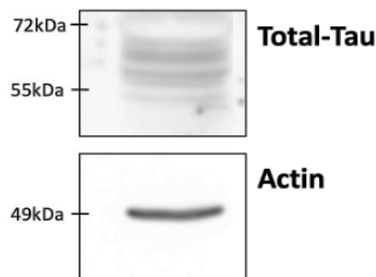


Figure 1'. *Tau isoforms detected in cells from the olfactory neuroepithelium.* Western blot analysis was performed on nasal swab samples using primary antibodies against Tau (D1M9X, 1:500; Cell Signaling Technology, 46687S) and actin (1:200,000; Sigma-Aldrich, A5441).

CHAPTER 1 : Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer's Disease

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Personal contribution: Everything except for the establishment of the diagnosis of the participants and samples used.

ABSTRACT

INTRODUCTION: Olfactory decline often precedes cognitive symptoms in Alzheimer's disease (AD) and is linked to tau pathology. Yet, whether tau accumulation start in peripheral olfactory structures or spread from central regions remains debated.

METHODS: We analyzed 34 *post-mortem* human olfactory bulb (OB) and 2 neuroepithelium (ONE) samples across Braak stages, combined with *ex-vivo* nasal swabs and standardized olfactory testing in 88 clinically characterized participants.

RESULTS: Tau accumulation appeared in the anterior olfactory nucleus layer of the OB from Braak stage III, spreading to peripheral layers only in late stages. Olfactory identification declined in the preclinical phase and was linked to central tau pathology. Discrimination worsened during the prodromal stage, while threshold impairment appeared only in dementia, reflecting the anatomical progression of tau pathology.

DISCUSSION: This anatomical–functional link supports a central-to-peripheral spread of tau pathology in the olfactory system, with stage-specific deficits suggesting targeted smell tests as early AD biomarkers.

BACKGROUND

AD is a mixed proteinopathy characterized by extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau [1, 2]. Both pathologies contribute to neurodegeneration and cognitive impairment [1], but NFT burden shows a closer correlation with cognitive decline [1, 3, 4] and more strongly predicts symptom onset [2, 5-7]. NFTs emerge through pathological tau transformation, in which excessive phosphorylation induces conformational changes that promote misfolding, oligomer formation, and subsequent aggregation [8]. These oligomers progressively assemble into paired helical filaments [1], which first appear as neuropil threads within axons and later evolve into pre-tangles in the perinuclear cell body [9]. With disease progression, these structures mature into NFTs that disrupt neuronal integrity, impair synaptic communication, and compromise network function [3]. Even after neuronal death, ghost tangles persist [9], highlighting their stability and underlining the role of tau aggregation as a progressive and self-perpetuating driver of AD pathology.

Tau pathology, widely regarded as the most reliable marker of AD progression, is believed to originate early in life in the locus coeruleus, later spreading to the transentorhinal cortex during Braak Stage I [10]. By Braak stage II, the OB, entorhinal cortex (EC), and hippocampus begin to exhibit pathological changes [10]. Notably, during Braak stages I-II, cognitive functions remain intact. However, olfactory dysfunction often arises in AD before the onset of overt cognitive impairment and mostly impacting odor identification ability [11-16]. This olfactory decline has been linked to pathological tau accumulation in the EC at early stages, and in the OB, more specifically in the anterior olfactory nucleus (AON) layer, around Braak stages II to III [17-20].

Despite the established correlation between olfactory dysfunction and AD progression, it is still unclear whether the olfactory system plays a primary role in the development of the disease. The "olfactory vector hypothesis" proposes that the olfactory system may serve as an entry

point for environmental or infectious agents that trigger cortical neurodegeneration [21-23]. Supporting this model, preventing the accumulation of pathological forms of tau in the ONE through its recurrent stripping in the mouse model expressing the human P301S mutant form of tau, markedly reduces pretangles-like tau pathology in the piriform cortex [24].

In humans, evidence for early tau pathology in the ONE is limited and inconsistent. A few studies have reported tangle-like profiles in the ONE tissue of AD cases [25-27], yet these observations were made predominantly in patients at advanced stages of the disease. Conversely, some studies reported little to no tau labeling in pathological conditions, with occasional staining also observed in non-AD or control individuals [28-31]. Contrasting conclusions drawn by these studies contribute to the uncertainty surrounding whether the human ONE exhibits tau pathology, particularly at the earliest stages of AD. Moreover, a recent study convincingly demonstrated tau propagation from medial temporal lobe structures toward the peripheral olfactory system [32], challenging the theory that tau pathology occurs in the ONE due to infectious or environmental agents spreads and eventually spreads to connected region. These opposing hypotheses nevertheless underscore the complexity and potential bidirectionality of tau spreading within the olfactory pathway, as well as possible differences between humans and mice.

Determining whether tau pathology originates in the olfactory cortex or peripheral olfactory structures is crucial for developing early interventions for AD. The primary objective of this study was to test the directionality of tau pathology propagation within the human olfactory system. To address this objective, we combined histopathological analyses of *post-mortem* olfactory tissues with *ex-vivo* nasal epithelial samples from individuals spanning the AD clinical continuum, together with olfactory assessments. This integrated approach enabled us to map the anatomical progression of tau pathology, supporting a trajectory from the central nervous system to peripheral olfactory structures. It also underscored the value of combining biological and functional measures to capture early disease dynamics.

METHODS

***Post-mortem* human samples**

Human *post-mortem* OB tissues were obtained from the Netherlands Brain Bank (NBB), with anonymized clinical summaries of the donor's medical record and histopathological brain analysis (**Table S1**). Two *post-mortem* human OB tissues from body donors at Braak stage II were obtained from UCLouvain body donation program (ethical approval: UCL-2020-355) (**Table S1**). In total, OB samples from 34 patients were used (F=20, M=14; 81.0 ± 15.1 years). All presented tauopathy ranging from Braak stage I to Braak stage VI (I: $n=3$, II: $n=5$, III: $n=5$, IV: $n=5$, V: $n=4$, VI: $n=12$). Braak staging was determined by the brain banks according to a widely accepted neuropathological standard [33] based on phosphorylated tau (P-tau) immunohistochemistry using the AT8 antibody (P-tau Ser202/Thr205). Assessments were performed by a single rater on sections from the hippocampus and EC, temporal associative cortex, and occipital cortex. Braak stages were interpreted as a continuum reflecting the progressive spread of neurofibrillary tangles from transentorhinal regions to the occipital cortex, with hippocampal involvement generally observed from stage II onward. For 15 patients, both frozen and fixed tissues were available; for 17 patients, only frozen tissues were available; and for 2 patients, only fixed tissues were obtained (**Table S1**). Frozen bulb samples were mechanically homogenized and split into two fractions for different analyses. Two *post-mortem* human ONE from body donors at Braak stage II were also obtained from UCLouvain body donation program (F=1, M=1; 92.5 ± 2.5 years, ethical approval: UCL-2020-355).

Participants recruitment for ex-vivo study

Participants were recruited through clinical neurology consultations and from clinical studies led by Prof. Bernard Hanseeuw and Prof.

Caroline Huart at Cliniques Universitaires Saint-Luc, the academic hospital of UCLouvain. This study was performed according to the Declaration of Helsinki and approved by the medical ethics committee of the academic hospital of UCLouvain (ethical approvals: UCL-2016-121 and UCL-2010-148). The 88 participants (F=45, M=43) provided written informed consent (**Table S2**). An oral questionnaire was run to exclude individuals meeting predefined exclusion criteria: known cause of olfactory disorder, acute or chronic rhinosinusitis, history of head trauma, participation in a clinical trial involving an AD treatment, or a medical history of major psychiatric conditions or other neurological disorders. The participants diagnosis described below, relied on an examination of amyloid and tau status with either Tau-PET imaging with [^{18}F]-MK6240 plus a 3DT1 MRI (n=37) [34], Amyloid-PET (n=57), cerebrospinal fluid (CSF) analyses (n=19) or blood plasma analyses (n=9). Clinical diagnosis were based on the Mini-Mental State Examination (MMSE) (n= 88) and a neuropsychological examination (n=57) which included testing of four cognitive domains (verbal episodic memory, executive functions, language and visuospatial functions), described in the supplementary methods.

Evaluation of amyloid and tau status

Amyloid positivity was determined by either CSF analysis, blood analysis or by amyloid PET with [^{18}F]Flutemetamol or [^{11}C]PIB as described previously [35, 36]. PET values were expressed on the Centiloid scale, blood plasma A β 42/ A β 40 and CSF A β 42 levels were measured with automated assays [35-37]. Participants were classified as amyloid positive (A+) if Centiloid >26, plasma soluble A β 42/A β 40 ratio ≤ 0.0465 or CSF A β 42 <437 pg/ml [36, 38-40]. Tau pathology was assessed with [^{18}F]MK-6240 PET (Lantheus Inc.), a second-generation tracer for tau tangles as described previously [41]. Radiosynthesis was performed at KULeuven and doses of 185 ± 5 MBq were administered intravenously. Ninety minutes post-injection, a 30-min dynamic acquisition was performed on a Philips Vereos

digital PET-CT. Images were reconstructed with the manufacturer's algorithm (attenuation, scatter, decay correction, TOF, PSF modeling, 1 mm reslicing). Tau PET scans were co-registered with a 3D-T1 MRI for visual analysis and the Braak-like stage was reported for each participant. Participants were classified as tau positive (T+) if the visual Braak-like stage was > 0 .

Neuropsychological evaluation

For the participants recruited through clinical studies, four domains were evaluated: episodic memory (FCSRT, French version [40]), language (Lexis Naming Test [41], Category and Letter Fluency Test), executive functions (Trail Making Test A and B [42], Luria's Graphic Sequences [43]) and visuospatial skills (Clock Drawing Test [44], CERAD praxis [45]). For each domain, three measures were z-scored and averaged to compute a global cognitive z-score [46]. Impairment was defined as $z < -1.5$ SD. Participants were considered cognitively impaired if at least one domain met this threshold; otherwise, they were classified as cognitively normal (CN). The MMSE test was also used for grading the cognitive state of the cognitively impaired participants [47].

Clinical classification of participants

Participants were classified as either cognitively normal (CN) ($n=47$, all cognitive domain z-scores ≥ -1.5 and MMSE > 24) or cognitively impaired (CI) ($n=41$, at least one cognitive domain z-score < -1.5). Among CN participants, 44 had available amyloid testing and were further subdivided into two subgroups with either normal ($n=29$, A-) or abnormal ($n=15$ A+) amyloid status, while 3 CN participants had no available amyloid or tau biomarker data. The A+ CN group comprises preclinical AD patients with either normal ($n=8$ T-) or abnormal ($n=7$ T+) tau-PET. To focus specifically on AD, only A+ CI individuals were included. A+ CI participants were further subdivided in two subgroups

based on disease severity with either prodromal AD (PR AD) ($n=28$) or AD dementia ($n=13$) (**Table 1** and **S2**).

Olfactory tests

Olfactory tests were performed using the Sniffin' Sticks test® (Burghart, Wedel, Germany) validated by [48]. This test includes three subtests yielding 3 scores: threshold (T), discrimination (D) and identification (I). Odors were delivered using pen-like devices with tips infused with an odorant solution. The tip was held 2 cm below the nose and moved slowly from the left to right nostril for over 3-4 seconds. The threshold test consisted of randomized triplets of pens numbered 1 to 16. In each triplet, only one pen contained the odorant (phenylethanol), with pen no. 1 containing the most concentrated solution. The odorant was serially diluted (1:2) down to pen n°16. For each triplet, the subject was asked to identify the odor-containing pen in a 3-alternative forced-choice (3-AFC) paradigm with eyes closed. The test starts with the lowest concentration, and a staircase paradigm is used, where two consecutive correct answers led to a lower concentration in the next triplet and one incorrect answer led to a higher concentration in the next triplet. This procedure is repeated until seven turning points are identified. The T score was the mean of the last four turning points in the staircase, ranging from 1 to 16. The discrimination test presented sixteen triplets in random order: two pens contained the same odor, while the third contained a different odor. Subjects identified the different odor in a 3-AFC paradigm, eyes closed. The D score was the number of correct responses (0 to 16). Sixteen pens were presented individually; for each, subjects were asked to identify the odor based on a four-alternative forced choice procedure from four illustrated options read aloud by the examiner. The I score was the number of correct identifications (0 to 16). The final TDI score was the sum of the three individual scores (range 1 to 48).

Nasal brushing and cell isolation

Nasal brushing was performed by a trained ENT specialist using two flocked swabs (Copan, FLOQSwabs™ 503CS01) introduced into both olfactory clefts at the level of the middle turbinate under endoscopic guidance. The ONE was exfoliated from both nostrils by rotation. One swab was placed in 1 mL of 4% PFA; the other in 1 mL chilled DMEM/F12 (ThermoFisher Scientific, Gibco™ 11039021) with 10% FBS (Cytiva, SV30160.03), 1% Penicillin/Streptomycin solution (Capricorn Scientific, PS-B), protease inhibitors (1:25; Roche, 11836170001), and phosphatase inhibitors (1:20; Roche, 4906837001). After vortexing for 15 s, the swab was removed from the fixative. The cell-containing solution was centrifuged 15 min at 300 x *g*, fixative discarded, pellet resuspended in PBS, and cytocentrifuged (Cytospin 4, ThermoFisher Scientific) for 10 min at 200 x *g* onto slides (Immuno-Cell, 61.100.59). The swab in culture medium was rubbed against another swab to detach cells. Samples were then divided into two aliquots before centrifugation at 4°C for 45 s at 20,630 x *g*. Supernatants were removed and cell pellets were frozen at -80°C.

Immunohistochemistry

Ten-micrometer sections of paraffin-embedded *post-mortem* OB tissues were prepared using a microtome (Microm Microtech, HM 340E). The sections were deparaffinized by incubating them in two successive xylene baths (5 min each), followed by two 2-min immersions in 100% isopropanol. Endogenous peroxidases were inactivated in 0.3% H₂O₂/methanol for 30 min. After rinsing with distilled water and delineation of tissue areas using a DakoPen (Sigma-Aldrich, Z377821), sections were washed three times for 10 min in TBS, then blocked for 1 h at room temperature (RT) in 10% normal goat serum (NGS) (Sigma-Aldrich, S26-M) and incubated overnight with primary antibodies (**Table S3**). Secondary antibody incubation was carried out for 30 min at RT. Negative controls omitted

primary antibodies. Sections were washed 3 times 10 min in TBS, incubated with streptavidin-HRP (Vector Laboratories, SK-4103) for 30 min, rinsed 3 times 10 min, and developed with DAB (Vector Laboratories, PK-7100): 2 min for PHF1/AT8, 1 min 30 s for AT270/OMP. Reactions were stopped in distilled water. Sections were dehydrated through graded isopropanol baths (70-100%, 4 × 2 min) and finally mounted with DPX (Sigma-Aldrich, 1.00579.0500). Visualization was conducted using a ZEISS Axioskop 40 microscope; images were captured with ZEISS ZEN Blue 2.5 and analyzed in ImageJ2 [49].

Immunofluorescence

For IF experiments, *post-mortem* OB paraffin blocks were sectioned at 5 µm. Sections were deparaffinized and rehydrated through a series of xylene, isopropanol, ethanol, and distilled water washes, then rinsed in 1× PBS. Antigen retrieval was performed by heating in boiling citrate buffer (10 mM, 0.1% Tween, pH 6) for two 5-min cycles, followed by a 20-min cooldown at RT. Permeabilization was performed in Tris-HCl buffer with 0.5% Triton X-100 for 1 h at 37°C. Following 1× PBS rinses, non-specific binding was blocked for 45 min at RT using 1× PBS supplemented with 0.1% Triton X-100, 0.21% BSA and 2.1% NGS. For *ex-vivo* nasal brushing cells, antigen retrieval was performed with two successive incubations in boiling citrate buffer (1 min followed by 3 min), followed by 30 min of permeabilization at RT. Blocking was then carried out for 1 h at RT in 1× PBS containing 0.1% Triton X-100, 0.21% BSA, and 2.1% normal horse serum (NHS). Samples were incubated overnight at 4°C with primary antibodies (**Table S3**). Negative controls omitted primary antibodies. The next day, samples were washed and incubated for 40 min at RT with secondary antibodies (**Table S3**). After final washes with 1× PBS, slides were mounted in fluorescence mounting medium (Agilent, S3023) containing 0.1 µg/mL DAPI (Sigma-Aldrich, D9542). Visualization was done using a ZEISS Axio Observer Z1 confocal microscope; images

were acquired with ZEISS ZEN Blue 2.6 and analyzed in ImageJ2 [49]. Low-magnification (2X) images were obtained with a Panoramic Confocal scanner (3DHISTECH).

ELISA

Post-mortem OB and *ex-vivo* smear samples were lysed in a buffer containing 150 mM NaCl, 20 mM Tris, 1% NP-40 (Sigma, 74385-1L), and 10% glycerol, supplemented with protease inhibitors (1:25, Roche, 11836170001) and phosphatase inhibitors (1:20, Roche, 4906837001). Mechanical lysis was performed using a syringe (Terumo Agani, 15438652; BD Plastipak, 303172). After centrifugation for 10 min at 20,000 x *g* at 4 °C, the supernatant was collected and protein concentration was determined using the Bradford assay. Phosphorylated tau at Thr181 (P-tau181) and total tau were quantified using commercial sandwich ELISA kits (ThermoFisher Scientific, KHO0631; KHB0041) per manufacturer's instructions. All assays were run in duplicate. Protein input was maximized, averaging 20 µg/well. Detection limits were 15.6 pg/mL for P-tau181 and 31 pg/mL for total tau. P-tau181 levels were normalized to total tau for each sample.

Statistical analyses

Figures and statistical analyses were performed using GraphPad Prism 10. Normality was evaluated using the Shapiro–Wilk test ($\alpha=0.05$). For two-group analyses, Student's *t*-test was used when normality was met; otherwise, the Wilcoxon test was applied. For multiple group comparisons, parametric one-way ANOVA was used for normally distributed data, otherwise the Kruskal–Wallis test was applied.

RESULTS

1. NFT-like accumulation accumulate in the *post-mortem* OB from Braak stage III onward

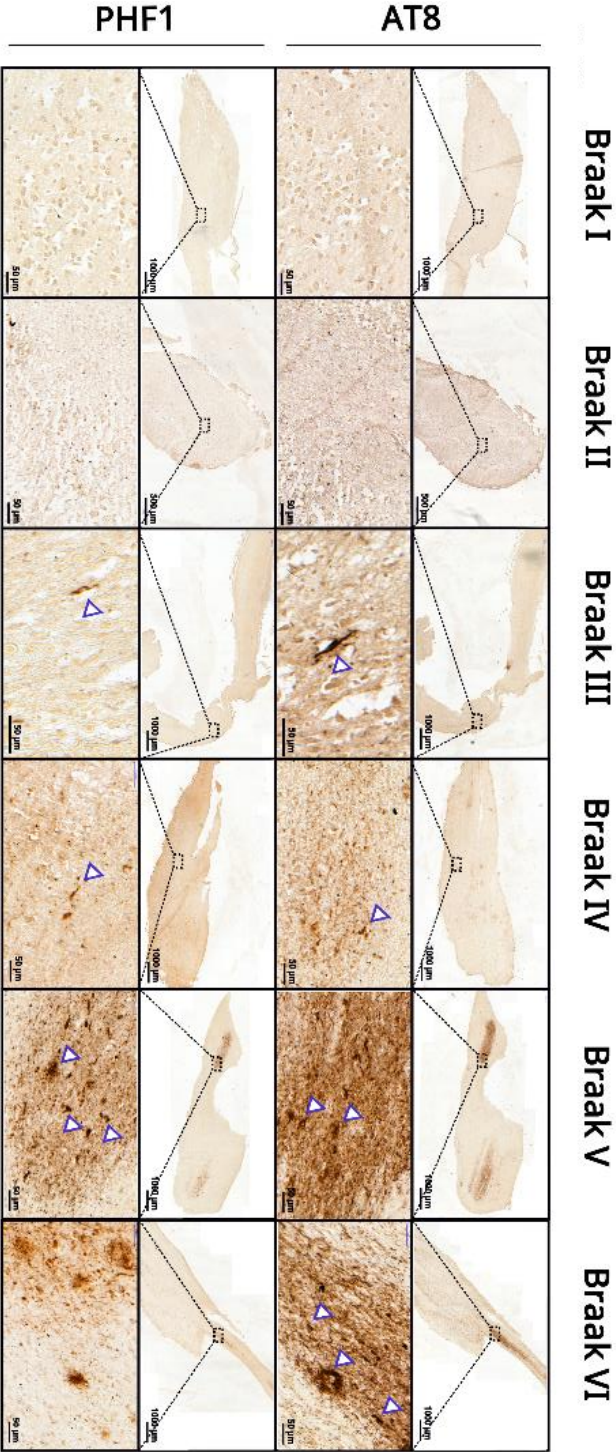
In the OB and AON, a relay structure that connects the bulb to the olfactory cortex, NFTs and neuropil threads have been previously reported to emerge from Braak stage II–III onward [17, 18]. Accordingly, using immunohistochemistry (IHC), we observed small amounts of NFT-like accumulation with AT8 (P-tau Ser202/Thr205) antibody in all thirteen *post-mortem* OB samples from donors with Braak stage III or higher, with their abundance progressively increasing at more advanced stages (**Fig. 1A** and **Fig. S1**). The flame-shaped accumulation characteristic of NFTs were however not observed in OB samples from patients at Braak stage I or II (**Fig. 1A** and **Fig. S1**). Similar observations were made with the PHF-1 antibody (P-tau Ser396/Ser404), a later-stage marker more specific to the mature forms of pathological accumulation and specifically highlighting flame-shaped NFT-like accumulation (**Fig. 1A**).

Consistent with previous studies [17, 50-54], we observed that, from Braak stage III onward, tau accumulation in the AON layer of the OB, which we identified using an anti-calbindin antibody – a marker of interneurons primarily located in the AON layer (**Fig. 1B**). In contrast, the outer layer of the OB, known as the olfactory nerve layer (ONL), identified using an antibody against the olfactory marker protein (OMP), remained unaffected at Braak stage IV (**Fig. 1C**). However, AT8-positive tau accumulation appeared by stage VI, indicating a delayed onset of NFTs in the ONL compared to the AON layer in AD patients (**Fig. 1D**).

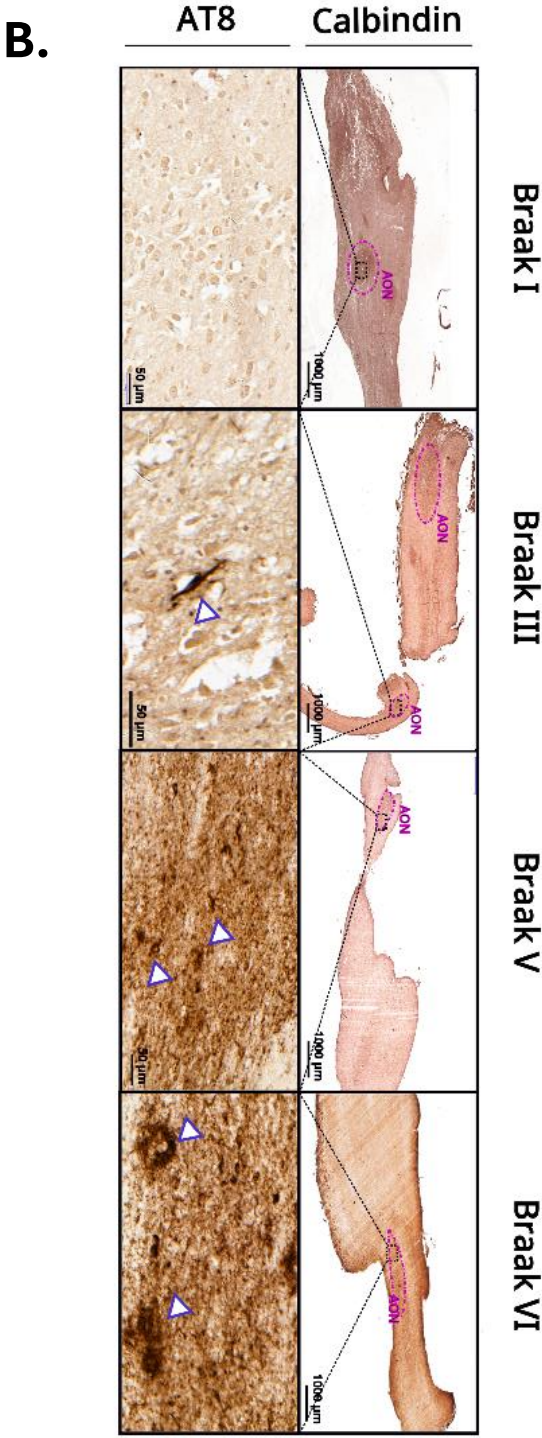
Together, our findings support the idea that although pathological tau emerge early in the AON layer, their accumulation in the ONL happens during the advanced stages of the disease.

Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer’s disease

A.



Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer’s disease



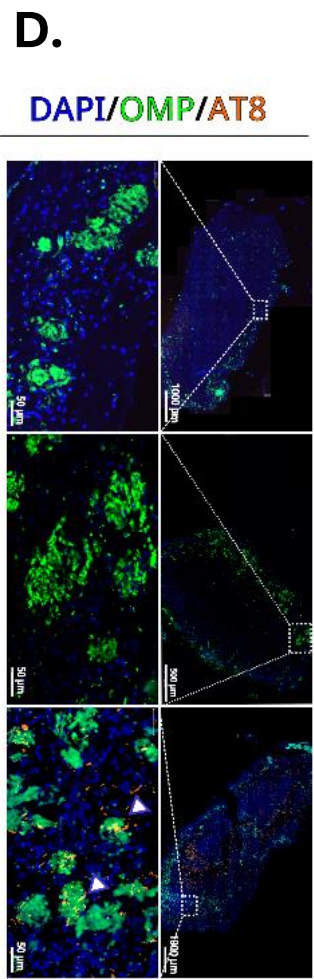
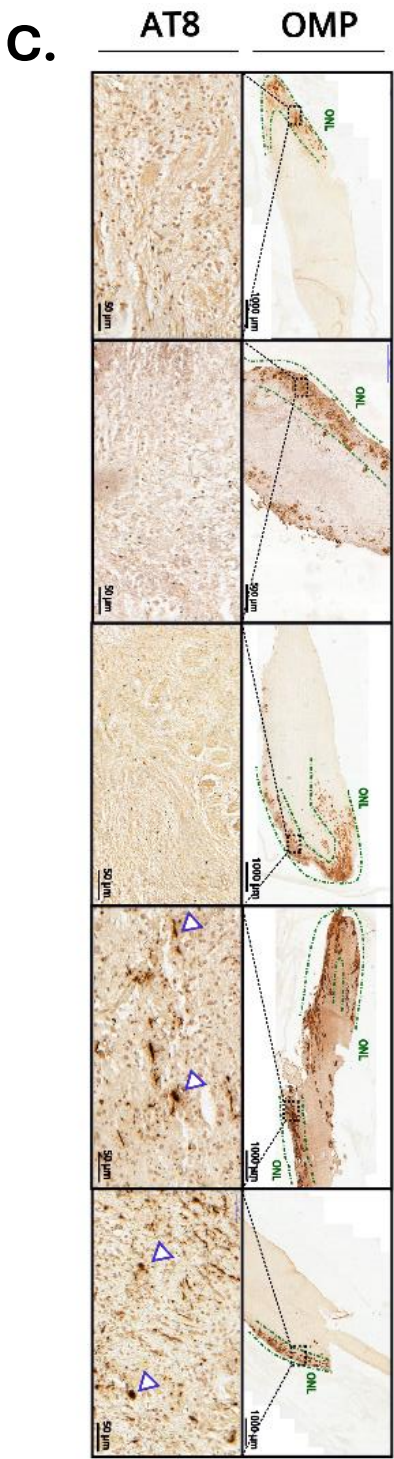


Figure 1. Distribution of NFT-like accumulation in human olfactory bulbs across Braak stages. (A) Representative pictures of paraffin sections immunostained for AT8 (Ser202/Thr205) or PHF-1 (Ser396/Ser404) in olfactory bulb (OB) samples from patients at the indicated Braak stages (n=6). Above: Low magnification view, scale bar: 500 or 1000 μ m, as indicated. Below: Higher magnification of the highlighted region, scale bar: 50 μ m. Flame-shaped NFTs (arrowheads) were not observed at Braak stages I or II. ((B) Above: The anterior olfactory nucleus (AON) region in the OB samples (n=4) was identified using calbindin immunolabeling (purple dotted lines), scale bar: 1000 μ m. Below: Higher magnification of the highlighted region, scale bar: 50 μ m. Arrowheads indicate NFT-like accumulation. Co-detection of the olfactory nerve layer (ONL) and NFTs in OB samples (n=5) from donors at indicated Braak stage using OMP and AT8 antibodies by IHC (C) or IF (D) (n=3) (lower panel with OMP in green, AT8 in red and DNA in blue). Above: Low magnification view, scale bar: 500 or 1000 μ m, as indicated. Below: Higher magnification of the highlighted region, scale bar: 50 μ m. Co-localization events are indicated by arrowheads.

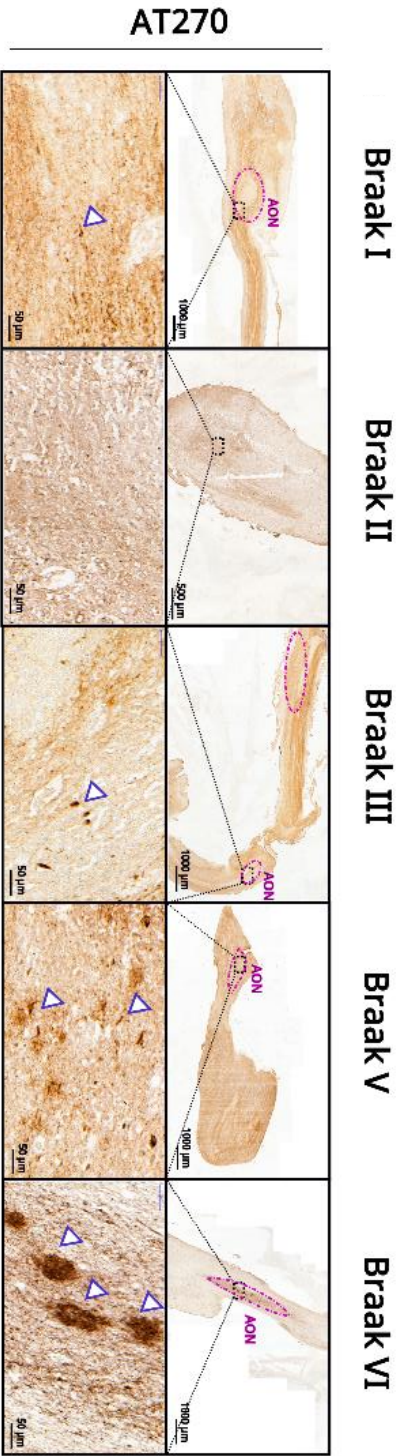
2. P-tau181-positive deposits accumulate in the AON from Braak stage I onward

Having found that tau accumulation appear in the OB of AD patients from Braak stage III, we next assessed the spatiotemporal progression of pathological tau filaments, specifically labeled by the AT270 antibody targeting P-tau181, a modification widely used as a quantifiable biomarker in human CSF and known to appear earlier in the progression of tau pathology than P-tau205 [49]. We detected tau deposits in the AON layer as early as Braak stage I, with a progressive increase in their accumulation at advanced stages (**Fig. 2A**). Notably, the early distribution of these accumulation appeared to be layer-specific. Specifically, in Braak I samples in which the earliest neuropil threads were observed in the inner layers of the olfactory bulb, we also noted the absence of these accumulation in the OMP-positive layers corresponding to the ONL (**Fig. 2B**). These findings support the notion that tau accumulation, from the formation of neuropil threads to the emergence of the first NFTs, initially develops in inner layers such as the AON before extending to more peripheral layers such as the ONL. Consistent with the AT8 and PHF1 stainings however, NFT-like accumulation only became detectable with these antibodies at Braak stage V and were found clustering in the AON layer (**Fig. 2A**). The results obtained by immunostaining were confirmed by ELISA as we

found a significant increase in the P-tau181/total tau ratios at advanced Braak stages (V/VI) compared with early stages (I/II) (**Fig. 2C**).

Together, this spatiotemporal progression of tau immunoreactivity across Braak stages supports a model in which the pathology progress from the AON layer to the rest of the OB.

A.



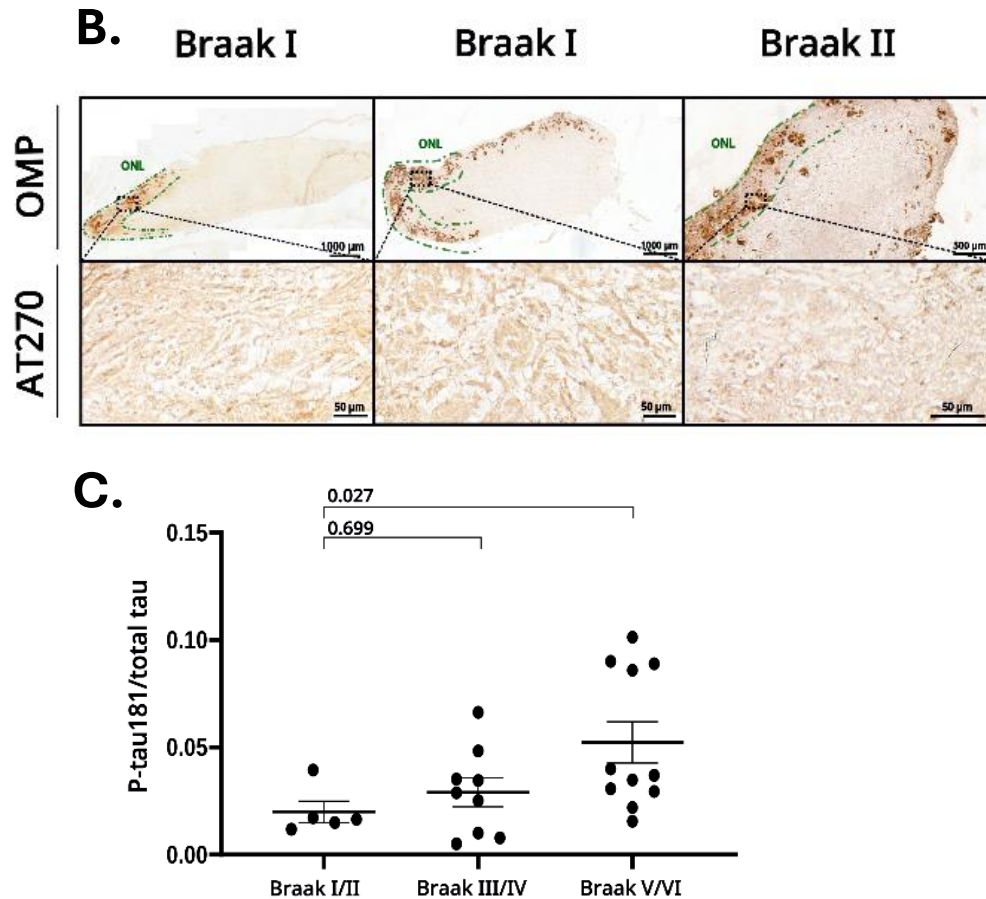


Figure 2. Detection of P-tau181 in human olfactory bulbs across Braak stages. (A) Representative pictures of paraffin sections immunostained with the AT270 antibody (P-tau181). Above: Low magnification view, scale bar: 500 or 1000 μ m, as indicated. The position of the AON is indicated with purple dotted lines. Below: Higher magnification of the highlighted region, scale bar: 50 μ m. Tau deposits appear as early as Braak stage I while NFT-like accumulation become detectable only from Braak stage V onward (arrowheads) in the OB samples ($n=5$). (B) Co-detection of the olfactory nerve layer (ONL) and neuropil threads accumulation in OB samples ($n=3$) from donors at indicated Braak stage using OMP and AT270 antibodies by IHC. Above: Low magnification view, scale bar: 500 or 1000 μ m, as indicated. Below: Higher magnification of the highlighted region, scale bar: 50 μ m. (C) Quantification of P-tau181 and total tau levels by ELISA in OB tissue extracts from patients at the indicated Braak stages ($n=25$). Mean $\pm$ SEM, Mann-Whitney statistical test.

3. NFT-like accumulation are not detected in exfoliated cells from the ONE in living humans

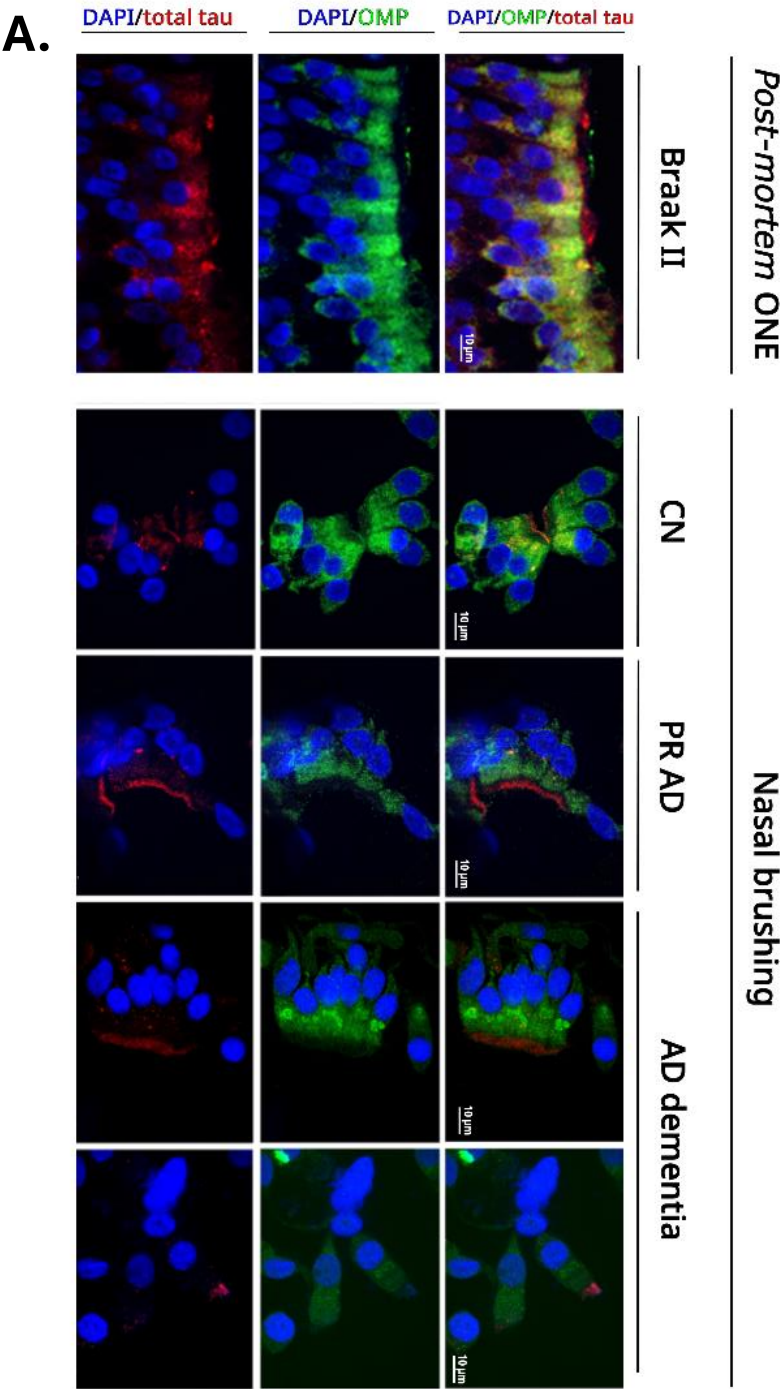
To further investigate the progression of tau pathology within the olfactory system, we next examined the presence of NFTs in the ONE. Previous studies on AD pathology in the ONE have primarily examined *post-mortem* tissues from individuals with late-stage dementia, limiting the ability to assess the presence of tau pathology in the ONE at earlier Braak stages [27,28]. Due to the limited availability of *post-mortem* ONE tissues, we adopted an *ex-vivo* approach, analyzing exfoliated cells collected from nasal brushing of living individuals. These samples were collected from a cohort representing the full clinical spectrum of AD, including amyloid-negative cognitively normal individuals (A- CN, $n=29$), as well as patients at preclinical stage, cognitively normal, but with amyloid pathology (A+ CN, $n=15$). This cohort also included cognitively impaired patients at the prodromal (PR AD, $n=28$), or dementia (AD dementia, $n=13$) stages of AD (**Table 1** and **S2**).

Category	A- CN	A+ CN	Prodromal AD	AD Dementia
Number of participants	29	15	28	13
Mean age	70,4	73,6	70,8	75,2
Sexe (F:M)	(15:14)	(8:7)	(13:15)	(8:5)
Amyloid testing (A)	A-	A+	A+	A+
Tau testing (T)	T-	T- / T+	T+	T+
Global cognitive Z-score (SD)	≥ -1.5	≥ -1.5	< -1.5	< -1.5
MMSE (/30)	>24	>24	>24	≤ 24

Table 1. Individuals enrolled in the study for nasal brushings and olfactory tests.
Summary of demographic and diagnostic data for individuals enrolled in the study, including clinically normal participants and patients at preclinical, prodromal, or advanced stages of dementia. Diagnoses were based on comprehensive clinical evaluation, neuropsychological testing, brain imaging (PET), blood samples and cerebrospinal fluid analysis when available. MMSE: Mini-mental state examination.

First, to determine whether nasal smears contain mature olfactory sensory neurons, we performed immunofluorescence against OMP, using *post-mortem* ONE tissue as positive control for the staining (**Fig. 3A**). Next, we tested a total of 26 nasal smear samples ($n=9$ CN, $n=9$ PRAD and $n=8$ AD dementia) and observed OMP staining in about 11 % of the collected cells (**Fig. 3A**), with a pattern closely resembling previously reported observations [50]. After validating the nasal brushing approach for studying the ONE, we proceeded to assess tau pathology. After validating the nasal brushing approach for studying the ONE, we proceeded to assess tau pathology. First, we confirmed the presence of total tau in the dendritic cilia of nasal smear cells, mirroring the distribution observed in the ONE tissue (**Fig. 3A**). However, AT8 immunoreactivity was absent in OMP-positive neurons collected *via* nasal smear, even in individuals with dementia, suggesting that NFTs do not form in these cells (**Fig. 3B**).

These findings indicate that NFT-like lesions do not form in olfactory sensory neurons, supporting the hypothesis that ONE is unlikely to serve as the entry point for tau pathology in humans.



Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer’s disease

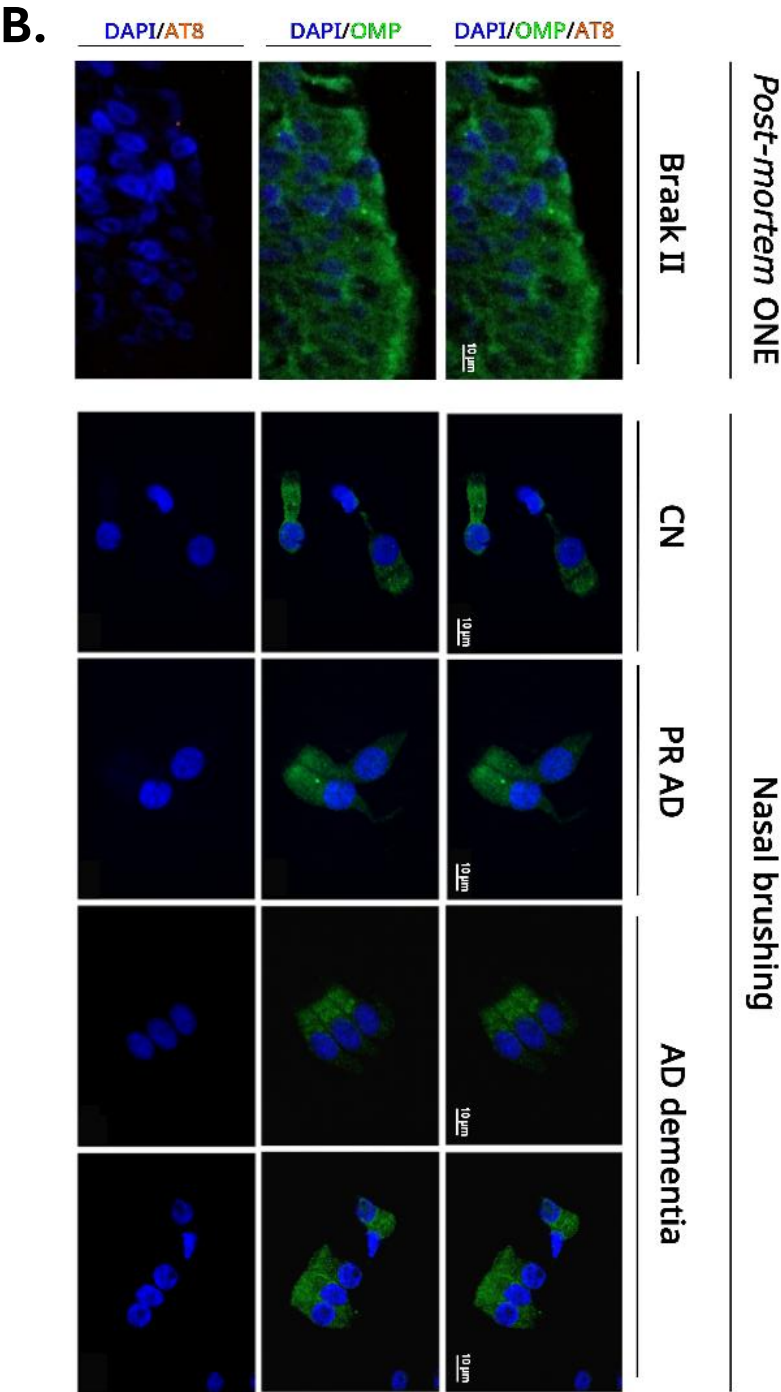


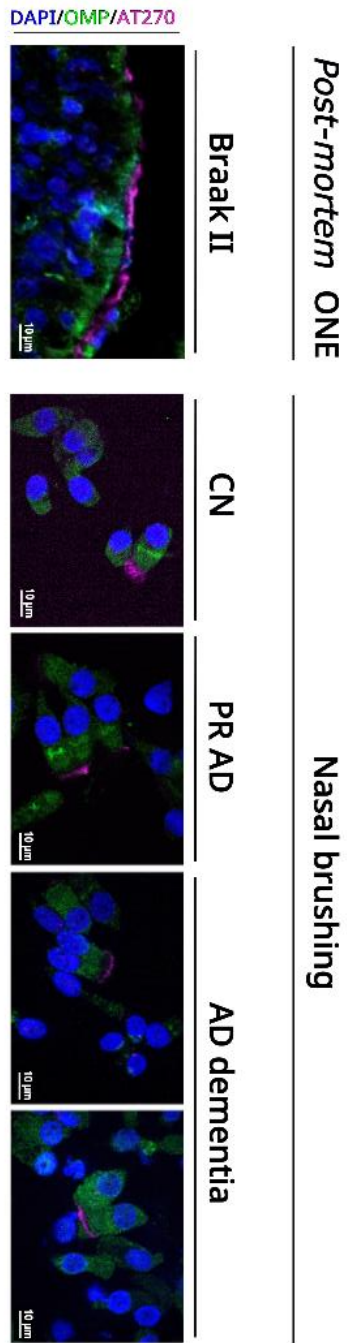
Figure 3. Detection of tau in human olfactory neurons from olfactory neuroepithelium and ex-vivo nasal smears. (A) Representative pictures of immunofluorescence staining using OMP (green) and tau (red) antibodies. DNA, stained with DAPI (blue), marks nuclei. Left: post-mortem ONE sample from a patient at Braak stage II ($n=1$); right: representative pictures of nasal smear cells ($n=4$) from amyloid-negative and cognitively normal (CN) donors or AD patients at the prodromal (PR AD) or the dementia stage of AD (AD dementia). Scale bar: 10 μm . **(B)** Same as A using OMP (green) and AT8 (red) antibodies. Scale bar: 10 μm .

4. Increased levels of P-tau181 in nasal smears from patients with AD dementia

Given the apparent lack of NFT-like lesions in nasal brushing, even in dementia patients, we next examined the samples for potential differential enrichment of P-tau181. We first attempted to measure P-tau181 levels in nasal smear extracts using an ELISA assay. While total tau was readily detectable, P-tau181 levels fell below the assay's detection threshold (**Fig. S2A-B**). We therefore turned to immunostaining, using *post-mortem* ONE tissue from a Braak stage II case as a positive control (**Fig. 4A**). To assess P-tau181 levels specifically in OMP⁺ cells within the nasal smears, we performed co-staining with AT270 (P-tau181) and OMP antibodies (**Fig. 4A**). For each sample, the ratio of OMP⁺/P-tau181⁺ cells was compared to the ratio of OMP⁺/total tau⁺ cells (**Fig. 3A and 4A**). Analysis of samples from CN ($n=9$), PR AD ($n=9$), and AD dementia patients ($n=8$) revealed a pattern consistent with that observed in the OB: a significant increase in tau phosphorylation in AD dementia cases ($p=0.037$), and a trend toward increased P-tau181 in the prodromal group ($p=0.23$) (**Fig. 4B**).

Together, these data support the results obtained with the OB samples and the hypothesis that human tau pathology does not start in the ONE.

A.



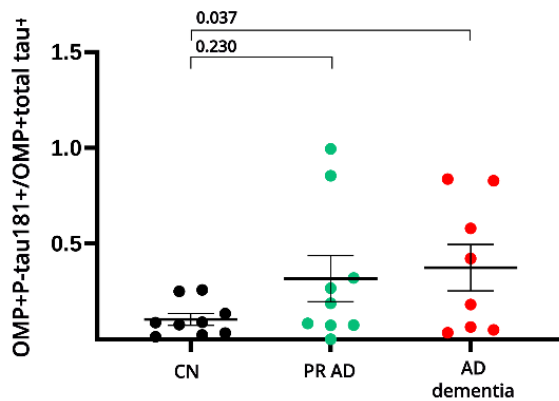
B.

Figure 4. Detection of P-tau181 in human olfactory neurons from olfactory neuroepithelium and ex-vivo nasal smears. (A) Representative pictures of immunofluorescence staining using OMP (green) and AT270 (P-tau181) (red) antibodies. DNA, stained with DAPI (blue), marks nuclei. Left: post-mortem ONE sample from a patient at Braak stage II (n=1); right: representative pictures of nasal brushing cells (n=4) from clinically normal (CN) donors or AD patients at the prodromal (PR AD) or the dementia stage of AD (AD dementia). Scale bar: 10 μ m. (B) Quantification of the ratio of OMP⁺/P-tau181⁺ to OMP⁺/tau⁺ cells across A- CN participants (n=9), prodromal AD patients (n=9), and AD dementia patients (n=8). Mean $\pm$ SEM, Unpaired t and Mann-Whitney statistical tests.

5. Olfactory identification declines in the preclinical stage, while detection threshold drops only in AD dementia

Early impairment in odor identification has been previously linked to tau accumulation in primary olfactory regions such as the piriform cortex, AON, amygdala, and EC [32]. Whether threshold performances, mainly thought to reflect peripheral olfactory function [56], get early affected in AD pathology is however less clear. To investigate this further and to gain additional insight into the direction of tau pathology spread, we evaluated olfactory function using the standardized Sniffin' Sticks test in an age-matched cohort encompassing various clinical stages of AD (**Table 1**). This test evaluates three distinct components of olfactory ability: identification

(I), but also threshold (T) and discrimination (D), each with a maximum score of 16.

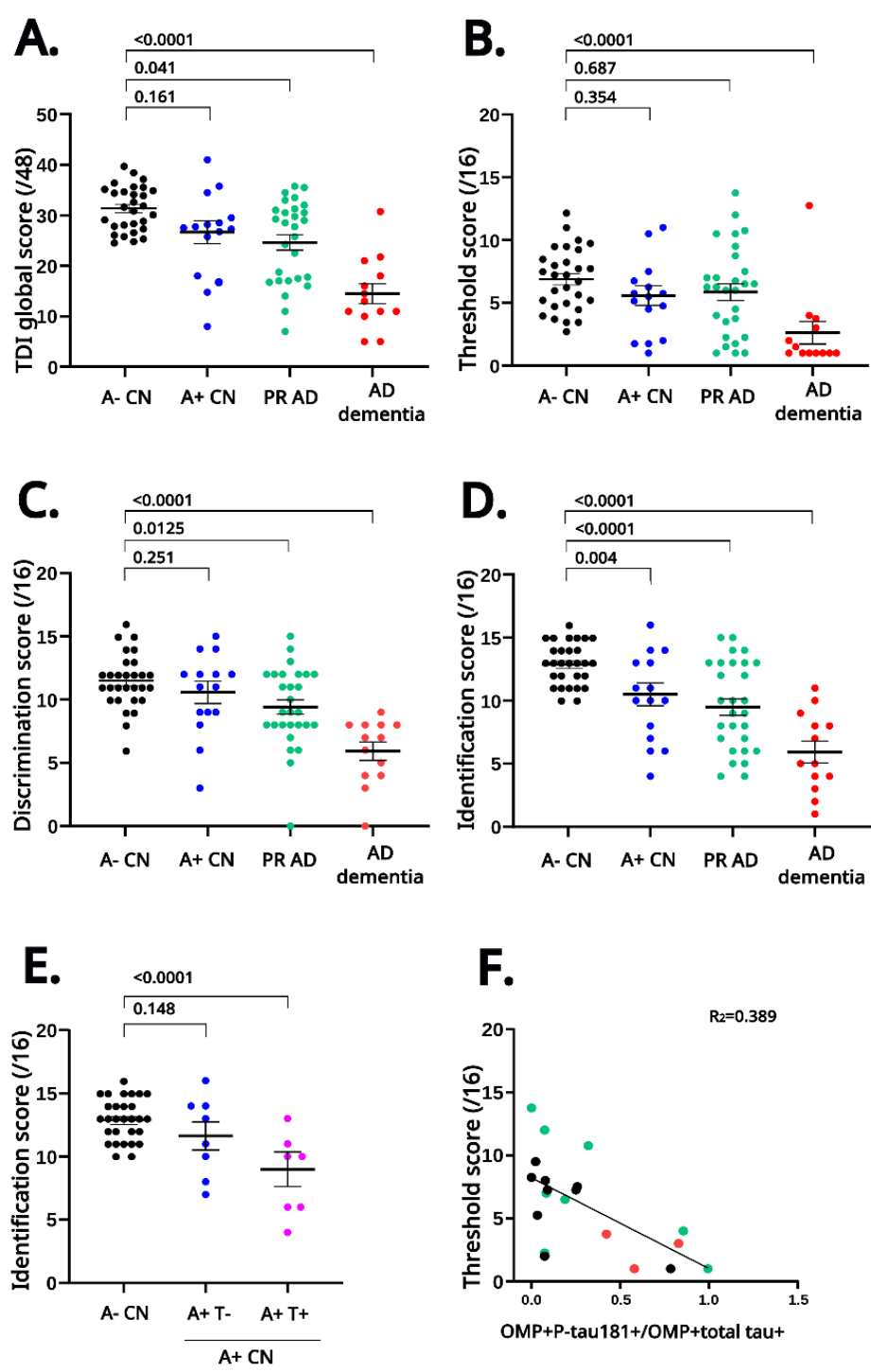
We found that the overall TDI score declined significantly at the prodromal ($p=0.041$) and dementia ($p<0.0001$) stage of AD compared to A- CN volunteers (**Fig. 5A**). When analyzing the three subcomponents individually, the threshold score showed a significant decline only at the dementia stage ($p<0.0001$) (**Fig. 5B**). In contrast, discrimination ability was already significantly impaired at the prodromal stage ($p=0.0125$) (**Fig. 5C**). Consistent with previous findings [15, 16, 52, 53], the identification score followed a different trajectory, with a significant reduction evident as early as the preclinical stage in A+ CN individuals ($p=0.004$), and continued to decline through the prodromal and dementia stages (**Fig. 5D**). A+ CN patients can be either negative or positive for tau pathology (**Table 1**). To further explore whether the early deficit in identification performance may be linked to tau pathology, we stratified A+ CN patients into two subgroups: those with a positive amyloid-PET, but negative tau-PET (A+T-) ($n=8$), and those with both amyloid and tau PET positivity (A+T+) ($n=7$). The results revealed a significant decline in identification scores exclusively in A+ T+ CN patients when compared to A- CN participants ($p<0.0001$), supporting an early association between olfactory dysfunction and tau accumulation in primary olfactory regions (**Fig. 5E**).

Consistent with the P-tau181 staining results, the decline in threshold performances was only seen at the dementia stage of AD, with no reduction at earlier stages (**Fig. 5B**). This prompted us to test whether the late decline in threshold performances may be related to the increased OMP⁺/P-tau181⁺ to OMP⁺/tau⁺ ratio that we observed in nasal smear cells in late disease stages (**Fig. 4A-B**). Consistently, we observed a trend toward an inverse correlation ($R^2=0.39$; $p=0.003$) between threshold scores and the ratio of OMP⁺/P-tau181⁺ to OMP⁺/tau⁺ cells in nasal smears (**Fig. 5F**), whereas the correlation

with discrimination scores was notably weaker ($R^2=0.15$; $p=0.087$)
(**Fig. S3**).

Together, these data support the hypothesis that early olfactory impairment in AD, marked by defective identification ability, stems from tau pathology in the central nervous system, which later spreads to the olfactory nasal epithelium and impacts detection threshold.

Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer’s disease



Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer's disease

Figure 5. Olfactory dysfunction correlates with tau pathology across Alzheimer's disease stages. (A) The composite TDI score was determined using the standardized Sniffin' Sticks test in n=29 A- CN (F=15, M=14; mean age=70.4yo) and n=15 A+ CN (F=8, M=7; mean age=73.6yo) participants, n=28 prodromal AD (PR AD) (F=13, M=15; mean age=70.8yo) patients, and n=13 patients at the dementia stage of AD (AD dementia) (F=8, M=5; mean age=75.2yo). (B) Threshold scores in the same cohort. (C) Discrimination scores in the same cohort. (D) Identification scores in the same cohort. (E) Identification scores in n=29 A- CN and n=15 A+ CN participants subclassified into n=8 A+ T- (purple) and n=7 A+ T+ (pink). (A) to (E): Mean $\pm$ SEM, Kruskal-Wallis and ordinary one-way ANOVA statistical tests. (F) Linear regression analysis between olfactory threshold scores and the ratio of OMP τ /P-tau181 τ to OMP τ /tau τ cells established in Fig. 4B on nasal smears.

6. Recognition of orange odor is significantly impaired from the preclinical stage

Earlier work using the UPSIT (University of Pennsylvania Smell Identification Test) assay of odor identification has associated pathological tau accumulation in the brain with impaired identification of certain specific odors [32]. Notably, the scent of orange was strongly associated with early tau deposition in olfactory regions during Braak stages II and III, while the scent of leather was linked to tau accumulation at Braak stages III to IV [32].

This prompted us to analyze participants' responses to each of the sixteen odors in the Sniffin' Sticks identification test (**Fig. 6A**). To focus on the most informative odors within the A+ CN group, we applied predefined selection criteria: (i) odors were required to have a correct identification rate of at least 80% in the A- CN group, thereby excluding odors with low baseline performance in cognitively normal individuals; (ii) identification performance in the A+ CN group had to show a reduction of at least 30% compared with the A- CN group; and (iii) this reduction had to be consistent across the AD continuum. Based on these criteria, identification of the orange scent was significantly impaired from the A+ CN preclinical stage ($p=0.0274$) (**Fig. 6B**). We further compared by stratifying the A+ CN group and observed that the significant alteration in orange identification was actually driven by A+ T+ patients ($p=0.0014$) (**Fig. 6C**). Following the same criteria, identification of leather and anise scents showed a delayed decline,

with impairments appearing only from the prodromal stage onward (**Fig. 6D-E**).

Collectively, the presence of selective odor identification deficits in the preclinical phase without accompanying threshold impairments, suggests that the olfactory system is more likely affected downstream in AD pathogenesis, rather than serving as an initial trigger. Notably, the early deficit in orange odor recognition, limited to T+ individuals within the CN group, further supports the involvement of tau pathology in this downstream effect.

RESULTS-CHAPTER I

Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer's disease

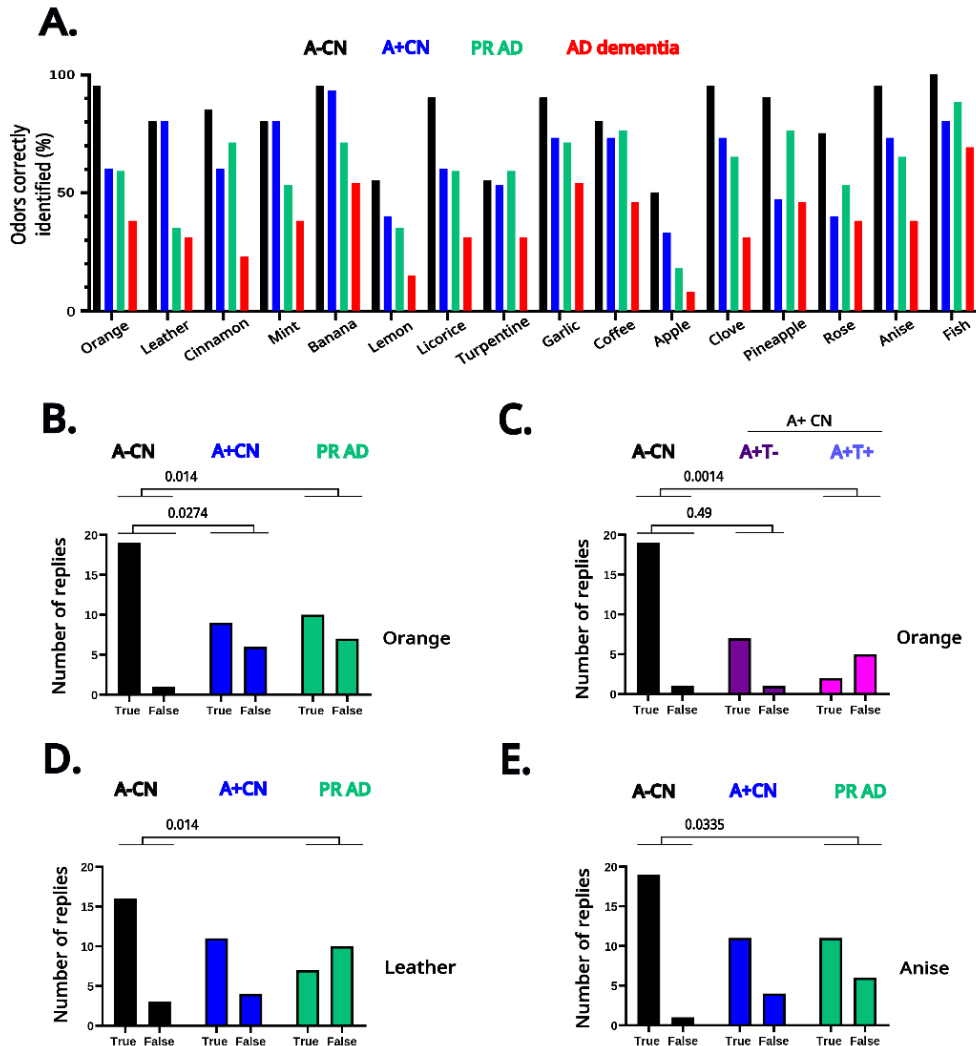


Figure 6. Differential impairment of odor identification across Alzheimer's disease stages. (A) Percentage of odors correctly identified by the participants for the sixteen odors in the Sniffin' Sticks test was calculated as a percentage of total true responses in $n=20$ A- CN (black) and $n=15$ A+ CN participants (blue), $n=17$ prodromal AD (PR AD) patients (green), and $n=13$ patients at the dementia stage of AD (dementia AD) (red). (B) Number of replies for the orange scent in $n=20$ A- CN (black), $n=15$ A+ CN (blue) and $n=17$ PR AD (green). (C) Number of replies for the orange scent in $n=20$ A- CN (black), $n=8$ A+ T- CN (purple) and $n=6$ A+ T+ CN (pink) participants. True: correct answer; False: wrong answer. (D-E) Same as (B) for the indicated scents. (B)-(E): Fisher's exact tests.

DISCUSSION

The reference model of tau propagation in AD, based on Braak staging, describes a “prion-like”, trans-synaptic spread from central to peripheral cortical regions [12, 59]. As olfactory deficits often precede the onset of cognitive symptoms, this has led some authors to propose an ascending prion-like propagation model initiated in peripheral structures such as the ONE and progressing toward the OB and cortex [16, 60, 61]. This model was supported by the observation that the ONE, directly exposed to the environment without the protection of a blood-brain barrier, is particularly vulnerable to toxic agents, infections, or other stressors [22, 57, 62]. However, this view has been challenged by other studies, reporting patterns consistent with a central-to-peripheral trajectory – namely, tau emerging in medial temporal and entorhinal regions and only subsequently involving olfactory cortices and peripheral olfactory structures [32, 63, 64]

Thus, the actual dynamics of tau pathology in the olfactory system remain controversial, notably because most available data derive from *post-mortem* tissues at advanced stages, making it difficult to infer the true chronology of spread [26, 27]. In this study, we combined cellular, molecular, and functional approaches to further explore whether the olfactory system plays a primary or secondary role in the etiology of AD.

We demonstrate that in AD, the OB exhibits tau accumulation, detected by AT8 immunoreactivity, from Braak stage III. Tau accumulation initially appears in the inner AON layer and only extends to the outer ONL at more advanced Braak stages (V-VI). In parallel, no tau accumulation were detected in the neuronal cell bodies of the ONE, either collected through nasal smears obtained from a cohort spanning the full AD continuum, or in two *post-mortem* ONE samples from Braak stage II patients. However, nasal smears from dementia-stage patients exhibited elevated P-tau181 levels without detectable accumulation.

Our findings align with previous reports indicating early NFT presence in the OB [17-20, 60, 61]. The sequential involvement of OB layers supports a temporal spread pattern. Given that the olfactory cortex is affected at Braak stage I [10], the most consistent interpretation is a central-to-peripheral propagation toward the ONE via retrograde transport [62]. However, it remains to be determined whether this progression reflects a prion-like mode of propagation or instead arises from differential cellular vulnerability across olfactory bulb layers.

These conclusions, however, contrast with previous reports describing tau pathology in the ONE, including tau-positive neuronal cell bodies [26, 27]. However, these observations are largely based on *post-mortem* samples obtained at advanced stages of AD, and no study to date has demonstrated the presence of tau accumulation in the ONE at very early disease stages. Moreover, when tau pathology is reported in the ONE, accumulation are predominantly observed in supporting structures, such as the lamina propria, rather than within the neuroepithelial layer itself [30]. In addition, studies in transgenic mouse models such as PS19 reported a propagation of tau from the periphery toward the central nervous system along the olfactory anterograde pathway [24], suggesting that, under specific experimental conditions, propagation may happen in both directions.

However, some limitations should be acknowledged in our study. The number of *post-mortem* samples was limited, and the ONE tissue was not represented across all Braak stages. In addition, the heterogeneous cellular composition and low neuronal content of nasal smears hindered precise biochemical quantification of tau. Notably, the quantification of P-tau181 was performed on a limited number of nasal smear samples, which should be taken into account when interpreting the statistical significance of the observed associations. Addressing these limitations in future work will allow a more complete characterization of tau pathology along the olfactory pathway.

Olfactory function was evaluated using the standardized Sniffin' Sticks test. In comparison to healthy controls, our results indicate that odor identification scores have declined significantly already at the preclinical stage of AD. In contrast, discrimination scores were impaired at the prodromal stage, while threshold scores only declined at the dementia stage. Our results suggest that functional impairments are closely linked to the development of tau pathology within the anatomical regions responsible for the respective olfactory functions. The decline in identification scores, which involves mainly cognitive function, was observed in preclinical A+T+ individuals, with no difference observed between A-T- healthy controls and preclinical A+T-, reinforcing that olfactory loss is more strongly linked to tau pathology rather than to amyloid burden [63-65]. This is consistent with previous reports showing that odor identification is typically the first olfactory function to be affected in the early stages of AD, a function primarily controlled by central olfactory regions [66, 67]. In particular, several studies have shown that loss of odor identification ability is associated with the presence of NFTs in the EC and the CA1 region of the hippocampus, both central olfactory structures affected from the earliest stages of the disease [11, 68, 69]. Furthermore, we observed an emerging correlation between threshold decline and the P-tau181/total tau ratio in nasal brushing, a measure reflecting neuroepithelial activity, providing novel evidence that peripheral olfactory function might also be associated with tau pathology.

Importantly, the temporal sequence of olfactory impairments parallels the anatomical progression of tau pathology inferred from the conclusions drawn from the analysis of OB and nasal smears. Interestingly, discrimination function, partly dependent on the OB [70, 71], is altered at the prodromal stage. This alteration appears to correlate with NFT-like accumulation detected in the bulb at the same stage in the immunostaining performed on the OB samples, in agreement with previous report of discrimination impairment at similar stages [58].

Beyond global scores, the analysis of individual odorants revealed selective impairments. Identification of orange scent was already reduced at the preclinical stage for T+ participants and continued to decline in prodromal and dementia stages. Leather and anise identification declined from the prodromal stage. This selective pattern, which confirms previous work for early affected orange scent in preclinical AD [32], supports a central origin of early olfactory deficits rather than a diffuse peripheral loss, which would be expected to affect identification of all odors. The specific early vulnerability of certain odorants, such as orange, raises questions about whether distinct neural circuits or receptor populations are selectively involved in the earliest phases of tau pathology spread.

From a translational perspective, the identification of specific odors and olfactory domains that are impaired at defined disease stages opens the possibility of developing targeted olfactory tests as complementary biomarkers for AD. In future work, longitudinal studies should investigate whether individuals with lower baseline olfactory performance, particularly in early-affected odorants, show faster clinical progression, as suggested by previous reports [15, 52, 72]. Such approaches, combined with comparative studies in other major neurodegenerative disorders, will be essential to determine the specificity and predictive value of olfactory testing for AD.

Several considerations should be noted. *Post-mortem* analyses of OB at Braak stage II were limited to a single case, and future studies including samples across the full range of Braak stages will be important to better capture disease progression. For smear analyses, age-matched comparisons could not be systematically performed given the number of participants recruited, although the mean age difference across groups was relatively small. Another limitation is that the z-scores used for cognitive stratification (CN, prodromal, dementia) were not adjusted for age, sex, or education. However, z-scores were derived from a single, homogeneous reference sample of 32 amyloid-negative individuals who remained cognitively stable over

an 8-year follow-up, [45] rather than from heterogeneous external normative datasets. This biologically and clinically well-characterized reference sample likely improved the consistency and comparability of standardized cognitive scores across domains and tests. In addition, cognitive stratification was reviewed by neurologists and neuropsychologists, incorporating functional status and all available clinical information. Finally, analyses in the A+ CN group were conducted in a relatively small cohort ($n=15$, 8 T- and 7 T+), which may reduce statistical power; nevertheless, these findings provide valuable preliminary observations that warrant replication in larger samples.

Finally, although patients with Lewy bodies disorders were excluded as rigorously as possible based on clinical criteria, the lack of sensitivity in vivo diagnostic tools means that the presence of undetected Lewy body pathology cannot be entirely ruled out. Given its known impact on olfactory function [73], this potential overlap may represent a limitation, as part of the observed olfactory deficits might not be exclusively attributable to AD pathology.

Overall, our results highlight the strong potential of olfactory testing for improving AD diagnosis and staging. Expanding odor panels linked to early misidentification may enhance diagnostic value. However, the absence of detectable tau accumulation in nasal smears from preclinical individuals suggests limited utility for early diagnosis. Future proteomic and transcriptomic analyses may help identify alternative early biomarkers in the ONE. Additionally, possible contributions of anterograde olfactory pathways to early OB involvement warrant further investigation, opening new perspectives for AD research and prevention.

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Supplementary Data for Chapter I

Supplementary methods

Neuropsychological evaluation

For the 57 participants who had a complete neuropsychological evaluation, four domains were evaluated: episodic memory (FCSRT, French version [1]), language (Lexis Naming Test [2], Category and Letter Fluency Test), executive functions (Trail Making Test A and B [3], Luria's Graphic Sequences [4]) and visuospatial skills (Clock Drawing Test [5], CERAD praxis [6]) (**Table S4**). Domain-specific composite scores were computed by averaging z-scores from three measures, calculated using an independent sample of 32 normal amyloid testing (A-) individuals who remained globally cognitively stable (as defined by a MMSE $\geq 26/30$) over an 8-year period (data collected in UCL-2010-412 study, see [46] for further details). These four domain-specific composite scores were averaged to produce a global cognitive composite score. To focus on Alzheimer's Disease (AD), abnormal amyloid testing (A+) participants were classified as cognitively impaired (CI) if at least one domain-specific composite score (memory, language, executive, visuospatial) was < -1.5 ; otherwise, they were considered cognitively normal (CN). Among CI participants, MMSE [47] and domain-specific cognitive composite scores were used to differentiate the prodromal stage from dementia. Individuals with an MMSE score $\geq 24/30$ and at least one domain-specific z-score < -1.5 were classified as having prodromal AD, whereas individuals with an MMSE score $< 24/30$ and either at least two domain-specific z-scores < -1.5 or a global cognitive composite score < -2 were considered as having dementia. Cognitive classifications were subsequently reviewed and confirmed by neurologists and neuropsychologists, considering patients' level of functioning in activities of daily living and all available information. Any discrepancies between initial classification and functional status or available clinical information were resolved through expert consensus. In the final classification, all patients diagnosed with dementia showed impaired functional abilities, whereas all individuals with prodromal AD

were globally independent in activities of daily living, consistent with standard diagnostic criteria [7-10].

Supplementary references

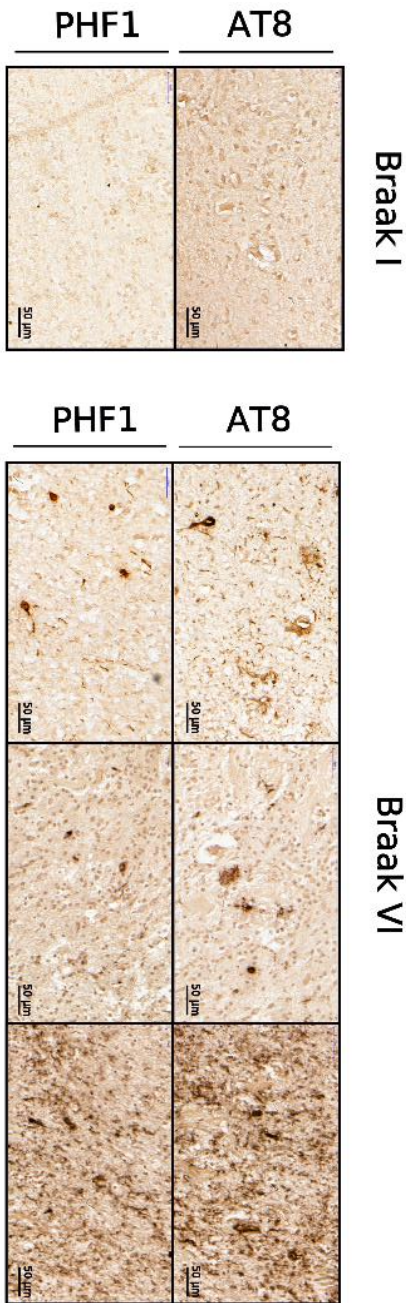
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Supplementary figures

Figure S1.



Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer's disease

Figure S1. Distribution of NFT-like accumulation in human olfactory bulbs. Representative pictures of paraffin sections immunostained for AT8 (Ser202/Thr205) or PHF-1 (Ser396/Ser404) in olfactory bulb (OB) samples from patients at Braak stage I (n=1) or VI (n=3). Scale bar: 50 μ m. Flame-shaped NFTs were not observed at Braak stage I.

Figure S2.

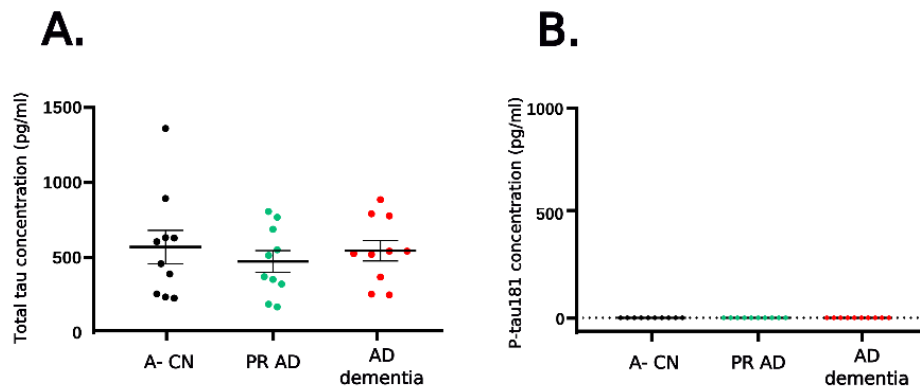


Figure S2. Quantification of total tau and P-tau181 in ex-vivo nasal smears. (A) Total tau concentrations were measured by ELISA in protein extracts from olfactory neuroepithelium cells collected by nasal brushing from three groups of participants: n=10 A- CN, n=10 prodromal AD (PR AD), and n=10 patients at the dementia stage of AD (AD dementia). (B) P-tau181 concentrations were below the detection limit in all samples.

Figure S3.

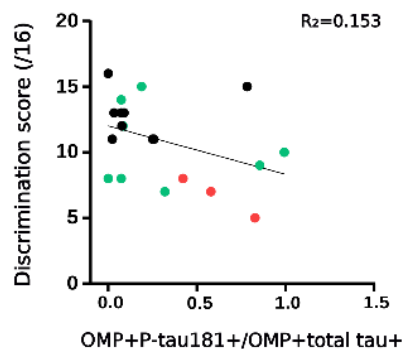


Figure S3. No correlation between the olfactory discrimination score and tau pathology in the olfactory neuroepithelium. Linear regression analysis between olfactory discrimination scores and the ratio of OMP⁺/P-tau181⁺ to OMP⁺/tau⁺ cells established in Fig. 4B on nasal smears.

Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer's disease

Supplementary tables

Table S1.

Postmortem sample	Sample type	Sexe	Age	Braak Stage	Sample storage	Analysis	Dementia	Cause of death
CTL1	OB	F	83	I	Fixed and frozen	IHC/ELISA	NO	Ileus, pancreatic cancer
CTL11	OB	M	84	I	Frozen	ELISA	NO	NA
CTL9	OB	M	73	I	Fixed and frozen	IHC/ELISA	NO	Collapse, followed by asystole. Probably cardiac death
CTL10	OB	M	89	II	Frozen	ELISA	NO	Pulmonary infection, urinary infection
CTL7	OB	F	99	II	Frozen	ELISA	NO	Euthanasia
CTL8	OB	F	102	II	Frozen	ELISA	NO	Old age
CTL12	OB	F	96	III	Frozen	ELISA	NO	Old age, cachexia, chronic UTI
CTL13	OB	M	92	III	Fixed and frozen	IHC/ELISA	NO	NA
CTL2	OB	F	90	III	Frozen	ELISA	NO	Possible infection, fever with unknown focus
CTL4	OB	F	95	III	Frozen	ELISA	NO	Ileus caused by tumor
CTL5	OB	F	102	III	Frozen	ELISA	NO	Ileus
AD17	OB	F	76	IV	Frozen	ELISA	YES	Decompensatio cordis
AD21	OB	M	85	IV	Frozen	ELISA	YES	Mesothelioma
ADLB5	OB	M	73	IV	Fixed and frozen	IHC/ELISA	YES	AD terminal stage. Possibly pulmonary embolism
CTL3	OB	F	102	IV	Frozen	ELISA	YES	Physical deterioration, heart failure
CTL6	OB	F	92	IV	Frozen	ELISA	YES	The flu

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AD16	OB	F	98	V	Frozen	ELISA	YES	Palliative sedation
AD18	OB	F	68	V	Frozen	ELISA	YES	Euthanasia
AD19	OB	F	94	V	Frozen	ELISA	YES	Pneumonia, dehydration
AD22	OB	M	64	V	Fixed and frozen	IHC/ELISA	YES	NA
AD11	OB	F	57	VI	Fixed and frozen	IHC/ELISA	YES	Dehydration, cachexia
AD14	OB	M	58	VI	Fixed and frozen	IHC/ELISA	YES	NA
AD20	OB	F	70	VI	Frozen	ELISA	YES	Gastric bleeding
AD23	OB	M	74	VI	Fixed and frozen	IHC/ELISA	YES	NA
AD6	OB	F	54	VI	Fixed and frozen	IHC/ELISA	YES	Exhaustion, cachexia
AD8	OB	M	65	VI	Fixed and frozen	IHC/ELISA	YES	Pneumonia by advanced dementia
AD9	OB	M	57	VI	Fixed and frozen	IHC/ELISA	YES	Aspiration pneumonia in end stage dementia syndrome
ADLB1	OB	M	56	VI	Frozen	ELISA	YES	Euthanasia
ADLB2	OB	F	69	VI	Fixed and frozen	IHC/ELISA	YES	Ileus
ADLB3	OB	M	83	VI	Fixed and frozen	IHC/ELISA	YES	Pneumonia by aspiration
ADLB4	OB	F	89	VI	Fixed and frozen	IHC/ELISA	YES	Ceased oral intake
ADLB6	OB	F	81	VI	Fixed and frozen	IHC/ELISA	YES	Dehydration, cachexia
UCL12	ONE	M	95	II	Fixed	IHC	NO	Grele occlusion
UCL15	OB and ONE	F	90	II	Fixed	IHC	NO	NA

Olfactory decline tracks central-to-peripheral spread of tau pathology in
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Table S1. Human postmortem samples information table. List of human olfactory bulb (OB) and olfactory neuroepithelium (ONE) samples used in this study. For each case, sex, age, Braak stage, type of storage (fixed and/or frozen), analysis performed (IHC and/or ELISA), presence of dementia and cause of death are indicated.

Table S2.

Participant	Sexe	Age	Diagnosis	PET tau
N41	M	60	AD dementia	ND
N05	M	71.0	AD dementia	+
N11	F	69.0	AD dementia	ND
N18	F	61.7	AD dementia	+
N52	F	76.3	AD dementia	ND
N72	F	92.0	AD dementia	ND
N78	M	87.6	AD dementia	ND
N83	F	73.4	AD dementia	ND
N90	F	71.5	AD dementia	+
N09	M	66.5	AD dementia	ND
N110	M	84.1	AD dementia	+
N15	F	82.1	AD dementia	ND
N16	F	82.5	AD dementia	ND
MCI1	F	67.0	Prodromal AD	ND
MCI10	F	59.0	Prodromal AD	ND
MCI11	M	83.0	Prodromal AD	ND
MCI2	F	66.0	Prodromal AD	ND
MCI3	M	59.0	Prodromal AD	ND

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MCI4	F	77.0	Prodromal AD	ND
MCI5	M	77.0	Prodromal AD	ND
MCI6	M	69.0	Prodromal AD	ND
MCI7	F	64.0	Prodromal AD	ND
MCI8	M	78.0	Prodromal AD	ND
MCI9	F	69.0	Prodromal AD	ND
N100	M	77.2	Prodromal AD	ND
N104	M	58.3	Prodromal AD	ND
N105	F	72.6	Prodromal AD	ND
N107	M	82.2	Prodromal AD	ND
N108	M	77.7	Prodromal AD	ND
N14	M	85.0	Prodromal AD	ND
N17	M	82.5	Prodromal AD	-
N23	M	76.9	Prodromal AD	+
N51	M	56.9	Prodromal AD	ND
N53	M	82.0	Prodromal AD	ND
N55	F	68.4	Prodromal AD	ND
N56	F	58.4	Prodromal AD	ND
N58	M	69.6	Prodromal AD	+
N86	F	53.0	Prodromal AD	-
N91	F	59.7	Prodromal AD	+
N97	F	75.5	Prodromal AD	+

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N19	F	77.7	Prodromal AD	+
CTL10	M	80.0	A- CN	ND
CTL15	F	65.0	A- CN	ND
CTL16	M	72.0	A- CN	ND
CTL3	F	66.0	A- CN	ND
CTL4	M	73.0	A- CN	ND
CTL5	F	65.0	A- CN	ND
CTL6	F	75.0	A- CN	ND
CTL8	M	71.0	A- CN	ND
CTL9	M	78.0	A- CN	ND
N102	M	61.5	A- CN	-
N109	M	61.7	A- CN	-
N20	F	77.7	A- CN	-
N24	M	67.4	A- CN	-
N26	F	80.0	A- CN	ND
N27	F	80.4	A- CN	ND
N28	F	52.6	A- CN	ND
N29	F	81.2	A- CN	ND
N30	M	77.05	A- CN	-
N31	F	61.6	A- CN	-
N33	M	90.2	CN	ND
N34	F	72.9	A- CN	ND

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N35	F	72.1	A- CN	ND
N36	F	75.1	A- CN	ND
N37	F	69.9	A- CN	ND
N38	F	74.6	CN	ND
N43	F	73.2	A- CN	ND
N45	M	74.2	CN	ND
N68	M	66.5	A- CN	ND
N70	M	69.0	A- CN	-
N74	M	68.5	A- CN	-
N76	M	69.8	A- CN	-
N89	M	59.4	A- CN	-
N21	F	76.1	A+ CN	+
N22	F	74.9	A+ CN	+
N61	M	68.4	A+ CN	-
N62	F	77.1	A+ CN	-
N64	F	74.2	A+ CN	+
N66	F	69.0	A+ CN	+
N67	M	77.4	A+ CN	-
N69	M	69.2	A+ CN	-
N71	M	68.9	A+ CN	+
N73	M	77.4	A+ CN	-
N79	F	60.0	A+ CN	-

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N87	F	84.0	A+ CN	+
N96	F	78.6	A+ CN	-
N99	M	77.4	A+ CN	+
N85	M	71.1	A+ CN	+

Table S2. Study Participants' Information table. Demographic and clinical characteristics of study participants, including sex, age, diagnosis, and tau PET status. PET tau status is indicated as positive (+), negative (-), or not done (ND).

Table S3.

Antibody	Target	Species	Dilution	Reference
PHF1	Phosphorylated Tau Ser396/Ser404	Mouse	1 : 300	Kindly provided by Peter Davies' Team
AT8	Phosphorylated Tau Ser202/Thr205	Mouse	1 : 500	Invitrogen, MN1020
AT270	Phosphorylated Tau Thr181	Mouse	1 : 500	Invitrogen, MN1050
D1M9X	Total tau	Rabbit	1 : 200	Cell Signaling, 46687S
OMP	OMP	Goat	1 : 500	Novus Biologicals, NB110-74751
Alexa Fluor 647	Anti-mouse	Donkey	1 : 1000	Invitrogen, A-31571
Alexa Fluor 594	Anti-rabbit	Donkey	1 : 1000	Invitrogen, A-21207
Alexa Fluor 488	Anti-goat	Donkey	1 : 1000	Invitrogen, A-11055

Table S3. Antibodies table. List of primary and secondary antibodies used in this study. For each antibody, the target, host species, dilution, and reference source are provided.

Table S4.

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Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer's disease

	<u>A-CN</u> (n=29)	<u>A+CN</u> (n=15)	<u>PR AD</u> (n=28)	<u>AD Dementia</u> (n=13)
MMSE (/30) – mean ± SD (n)	29.1±0.8 (n=29)	28.7±1.0 (n=15)	26.8±1.8 (n=28)	19.5±2.8 (n=13)
FCSRT SUM FR (/48) – mean ± SD (n)	33.7±4.9 (n=20)	32.5±3.9 (n=15)	19.6±7.2 (n=23)	14.8±7.5 (n=5)
FCSRT SUM TR (/48) – mean ± SD (n)	47.0±1.5 (n=20)	46.3±2.6 (n=15)	40.4±4.5 (n=23)	34.2±12.9 (n=5)
FCSRT Delayed FR (/16) – mean ± SD (n)	13.1±2.2 (n=20)	12.9±2.1 (n=14)	7.8±3.2 (n=22)	7.2±4.9 (n=4)
TMT B-A Time – mean ± SD (n)	45.5±28.2 (n=20)	43.2±27.8 (n=15)	135.2±133.7 (n=24)	173.1±98.3 (n=10)
TMT B-A Errors – mean ± SD (n)	0.6±1.1 (n=20)	0.1±1.0 (n=15)	1.3±1.9 (n=24)	1.0±1.4 (n=10)
Luria graphical series (/32) – mean ± SD (n)	28.9±2.1 (n=20)	28.7±2.1 (n=15)	23.7±4.0 (n=23)	17.7±8.1 (n=8)
Category Fluency – mean ± SD (n)	39.1±9.6 (n=20)	37.9±7.0 (n=15)	27.7±10.2 (n=25)	19.0±7.8(n=9)
Letter Fluency – mean ± SD (n)	25.5±7.8 (n=20)	26.9±6.2 (n=15)	19.2±6.4 (n=25)	15.2±8.4 (n=9)
Naming Lexis 64 (/64) – mean ± SD (n)	60.6±2.2 (n=19)	58.4±2.6 (n=15)	55.5±8.3 (n=20)	51.2±4.9 (n=9)
Clock Drawing (/8) – mean ± SD (n)	6.8±1.2 (n=20)	6.5±1.6 (n=15)	6.6±1.3 (n=25)	5.3±2.9 (n=10)
Clock Copy (/10) – mean ± SD (n)	9.9±0.4 (n=20)	9.6±1.3 (n=14)	9.5±0.9 (n=23)	8.3±2.3 (n=7)
CERAD Figure Copy (/11)– mean ± SD (n)	10.6±0.5 (n=20)	10.5±0.6 (n=15)	10.1±1.2 (n=24)	8.4±1.7 (n=9)
MEMORY Composite – mean z-score ± SD (n)	0.25±0.6 (n=20)	0.09±0.6 (n=15)	-2.1±1.6 (n=18)	-5.7±0.8 (n=4)
EXECUTIVE Composite – mean z-score ± SD (n)	0.2±0.6 (n=20)	0.3±0.4 (n=15)	-1.2±1.8 (n=17)	-2.3±1.2 (n=4)

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LANGUAGE Composite – mean z-score $\pm$ SD (n)	0.35 $\pm$ 0.7 (n=20)	0.3 $\pm$ 0.7 (n=15)	-0.6 $\pm$ 0.9 (n=18)	-2.1 $\pm$ 0.4 (n=4)
VISUOSPATIAL Composite – mean z- score $\pm$ SD (n)	0.12 $\pm$ 0.5 (n=20)	-0.2 $\pm$ 0.8 (n=14)	-0.4 $\pm$ 1.0 (n=18)	-2.9 $\pm$ 2.5 (n=4)
GLOBAL COGNITIVE Composite – mean z- score $\pm$ SD (n)	0.22 $\pm$ 0.4 (n=20)	0.08 $\pm$ 0.4 (n=15)	-1.1 $\pm$ 0.8 (n=18)	-3.3 $\pm$ 0.7 (n=4)

Table S4. Complete neuropsychological examination. The table presents mean and standard deviation (SD) for each cognitive score in each group. The number of data available for each test is specified in brackets (n). Missing data are due to some participants not having completed certain tests or, in the case of the most severely affected patients, being unable to complete the test.

Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

Preamble chapter II: Nasal swabbing has previously been validated in our laboratory as a minimally invasive approach to access and analyze cells from the olfactory neuroepithelium. In the first project, the sampling procedure specifically targeted the upper region of the nasal cavity in order to collect cells from the olfactory neuroepithelium, a specialized sensory tissue involved in odor detection. This tissue is anatomically restricted to the superior part of the nasal cavity and is characterized by the presence of olfactory sensory neurons, sustentacular cells, basal stem cells, and Bowman's gland-associated cells. Using this approach, we demonstrated that neuronal cells could be collected directly from living individuals and studied *ex vivo*, thereby providing access to cellular alterations associated with neurodegeneration and aging.

Building on this methodological basis, the second project relied on the same nasal brushing approach but focused instead on the respiratory epithelium. In contrast to the olfactory neuroepithelium, the respiratory epithelium is distributed throughout a larger part of the nasal cavity and mainly contributes to airway protection, mucociliary clearance, and immune defense. It is primarily composed of ciliated epithelial cells, goblet cells, and basal cells, making it structurally and functionally different from the neuronal olfactory tissue. Because of its broader anatomical distribution and easier accessibility, respiratory epithelial sampling is technically less complex and provides larger amounts of cellular material.

This methodological advantage was used to investigate senescence-associated markers *ex vivo* in human respiratory tissues. Indeed, cellular senescence has mainly been studied *in vitro*, particularly in fibroblast cultures, from which most classical senescence markers have been defined. However, these markers may not fully reflect senescence processes occurring in human tissues, since each tissue presents specific cellular compositions, functions, and biological constraints. Studying senescence-associated markers directly in human respiratory epithelial cells therefore represents an opportunity to identify biological alterations that may contribute to or promote aging processes in a more physiologically relevant context.

In addition to nasal respiratory epithelial samples, the project was extended to the analysis of post-mortem lung tissues obtained from a relatively large

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

cohort. Both the nasal respiratory epithelium and the lung belong to the respiratory system and are therefore biologically related tissues. Combining these two complementary tissue sources allowed a broader investigation of senescence-associated alterations within the human respiratory tract and contributed to a better characterization of markers potentially involved in biological aging.

Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CHAPTER 2 : Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

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Personal contribution refers to experiments performed on the nasal brushing samples. Lungs samples experiments, analyses and conclusions were performed by my fellow co-first authors.

ABSTRACT

As the global population grows older, cellular aging plays a central role in the increasing prevalence of age-related diseases. Its key features include telomere shortening and impaired proteostasis, often associated with DNA damage-induced transcriptional stress. In the lungs, epithelial aging plays a key role in the development of idiopathic pulmonary fibrosis (IPF), a fatal and progressive disease. While telomere dysfunction-induced foci (TIF) are well-documented in IPF lungs, their presence during normal human lung aging remains unclear. Using lung tissue from organ donors aged 16-88, we found a linear decline in telomere length (TL) with age. However, TIF frequency only significantly increased in the lung epithelium at very advanced ages, coinciding with the sharp rise in IPF prevalence in individuals over 75. Senescence markers such as p16 were however markedly lower in aged lungs compared to IPF lungs, suggesting that additional stressors contribute to IPF pathogenesis. To extend our analysis to the upper respiratory tract, we examined nasal epithelial cells obtained through brushing and found that, despite evidence of telomere shortening, TIF were infrequently observed even in individuals over 80 years of age. In contrast, markers of proteostasis disruption, including increased senescence-associated β -galactosidase (SA- β -Gal) activity and lysosomal content, were evident from age 40 and correlated with olfactory decline. These results suggest that, while age-related TIF induction in the aging lung epithelium may contribute to IPF development, telomere dysfunction is unlikely to be a primary driver of cellular senescence in the upper airway, where proteotoxic stress may play a more central role.

Keywords: aging, telomere, nasal epithelium, lung, cellular senescence, proteostasis decline, IPF

INTRODUCTION

Aging is the primary global risk factor for disease and death, marked by a progressive loss of physiological function that varies across organs. Aging of the human respiratory tract increases vulnerability to lung diseases, diminished olfactory function, and viral infections. Identifying the hallmarks of respiratory tract aging may facilitate the development of novel therapeutic interventions to lower their incidence.

The discovery of five core hallmarks of aging – genomic instability, telomere shortening, epigenetic changes, loss of proteostasis, and impaired macroautophagy (Lopez-Otin et al. 2023) – has significantly advanced our understanding of the aging process. However, a central challenge in aging research remains: identifying which of these hallmarks act as primary drivers of cellular aging. This question is further complicated by the fact that cellular senescence is heterogeneous, with aging hallmarks displaying organ-specific and time-dependent patterns (Cohn et al. 2023; Schaum et al. 2020), suggesting that there is no universal answer.

During the past decade, proteotoxic stress has emerged as a potential central factor in cellular senescence induction (Ding et al. 2025; Kaushik and Cuervo 2015; Lopez-Otin et al. 2013, 2023). With age, misfolded and damaged proteins accumulate, in part due to increased reactive oxygen species (ROS) production. Combined with a decline in lysosomal function, this leads to elevated lysosomal content in aging cells evidenced by the heightened activity of senescence-associated β -galactosidase (SA- β -Gal) (Dimri et al. 1995). The age-related decline in macroautophagy efficiency further impairs the clearance of damaged organelles, such as mitochondria, exacerbating ROS levels and cellular damage. *In vivo* evidence for the role of defective autophagy in aging comes from studies showing that restoring autophagy in aged mouse livers improves mitochondrial function and restores hepatic health (Zhang and Cuervo 2008). Additionally, recent research links proteostasis loss to DNA damage-induced transcriptional stress, resulting in widespread gene expression downregulation in aging cells (Gyenis et al. 2023; Stoeger et al. 2022).

Telomere deprotection, characterized by the formation of telomere dysfunction-induced foci (TIF), results in sustained activation of the DNA damage response (DDR), ultimately leading to irreversible cell cycle arrest

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and the induction of cellular senescence (Fumagalli et al. 2012; Hewitt et al. 2012). While telomere dysfunction, observed in cells from individuals with telomere biology disorders (TBDs) or during replicative senescence in cultured fibroblasts, can clearly trigger senescence (Rossiello et al. 2022), it remains unclear whether TIF formation plays a role in normal physiological aging. Although age-associated telomere shortening is well-documented in human tissues (Everaerts et al. 2018; Takubo et al. 2010), it is insufficient on its own to induce telomere dysfunction and drive cellular senescence (Rossiello et al. 2022).

Lung epithelium aging is a major risk factor for idiopathic pulmonary fibrosis (IPF). IPF is a fatal disease marked by cellular senescence and progressive lung scarring due to abnormal extracellular matrix accumulation, with prevalence rising sharply in individuals over 75 years of age (Hewitt et al. 2025; Schneider et al. 2021). Lung cells from patients with pulmonary disease, such as IPF, exhibit a higher frequency of TIF (Birch et al. 2015; Schafer et al. 2017). Supporting a causal link between telomere dysfunction and the onset of IPF, individuals with germline mutations in telomere maintenance genes develop TBDs that are frequently associated with an earlier onset of IPF (Armanios et al. 2007; Cronkhite et al. 2008; Revy et al. 2023; Rossiello et al. 2022). While TIF have occasionally been reported during physiological aging of the human lung (Birch et al. 2015; Schafer et al. 2017), it remains uncertain whether their frequency increases with age and contributes to IPF development. This uncertainty is partly due to the absence of data from non-IPF lung samples of individuals over 80 years old in these studies. The “second hit” hypothesis proposes that IPF arises from external insults acting on an already compromised and aging alveolar epithelium (Hewitt et al. 2025). Whether age-related TIF could represent the initial “first hit” in this model remains to be elucidated.

The nasal epithelium acts as a critical first barrier against inhaled pathogens, allergens, and irritants, and plays a central role in the development of both acute and chronic airway diseases (Balazs et al. 2022). Aging of the upper respiratory tract is closely linked to a decline in olfactory function (Doty and Kamath 2014; Doty et al. 1984) and was also recently shown to modulate the nasal epithelial responses to SARS-CoV-2 infection (Woodall et al. 2024). While studies using primary nasal epithelial cultures have reported a possible adaptive response to elevated oxidative stress in cells from older individuals

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(Balazs et al. 2022), the molecular mechanisms underlying aging in the upper airway remain largely unexplored.

In this cross-sectional study, we evaluated markers of cellular senescence in lung tissues from organ donors across a broad age range, as well as in freshly collected nasal brushing samples. Although telomere shortening was clearly evident in both tissue types, TIF abundance in the lung epithelium only significantly increased in donors aged 75 and older, reaching levels comparable to those found in IPF lungs. In contrast, nasal epithelial cells showed minimal age-related changes in TIF frequency. However, signs of proteostasis disruption, including elevated SA- β -Gal activity and increased lysosomal content, emerged as early as age 40 and were associated with olfactory decline. These findings suggest that although age-related telomere dysfunction in the lung epithelium may contribute to IPF pathogenesis, it is unlikely to serve as a universal trigger of cellular senescence. Rather, proteotoxic stress appears to play a more prominent role in driving age-associated tissue dysfunction in the upper respiratory tract.

METHODS

Lung samples

Unused or declined lungs for transplantation at University Hospital Leuven (Belgium) were obtained from a total of 20 donors (6 females and 14 males, 16-83 years) (Table S1). Lungs affected by emphysema were excluded. Collection of donor lungs for research was performed in accordance with Belgian legislation and approved by the local ethics committee (S59648, S61653). Lungs with IPF (n = 12, 3 females and 9 males, 53-66 years) were obtained from patients undergoing lung transplantation at University Hospital Leuven (Belgium) (Table S1). IPF was diagnosed through dynamic multidisciplinary discussion according to ATS/ERS guidelines (Raghu et al. 2018). Written informed consent was obtained from all patients, and the study was approved by the local hospital ethical committee (S52174). Lungs were collected as previously described (Verleden et al. 2021). Briefly, lungs were air-inflated immediately after resection, snap-frozen in liquid nitrogen fumes, and subsequently sectioned transversally into 2-cm slices stored at -80°C . For immunohistochemistry, selected cores were formalin-fixed and paraffin-embedded (FFPE). Frozen tissue samples were incubated in 10% formalin for 24 h at room temperature before processing with a Leica TP1020 automated tissue processor (Leica Biosystems, Wetzlar, Germany). Samples were then carefully oriented transversally and embedded in paraffin using a Leica Histocore Arcadia system. Additionally, peripheral wedge biopsies were collected from n = 13 control donor lungs (7 females and 6 males, 28-88 years) prior to implantation (Table S1) (Van Slambrouck et al. 2025). Biopsies were taken from the lingula of the left upper lobe, in accordance with institutional ethical approval (S67052). The staple line was removed and four small pieces were promptly placed in cryotubes and frozen on dry ice before storage at -80°C .

Nasal brushing sample collection

Healthy volunteers (n = 213, 117 females and 96 males, 2-97 years) were recruited from three independent clinical studies, all approved by the local ethics committee (Comité d'Ethique Hospitalo-Facultaire Saint-Luc–UCLouvain) under the following references: 2016/21MAR/121, 2021/10NOV/467, and 2022/07FEV/046 (Table S2). Individuals with TBD (n = 11, 7 females and 4 males, 18-62 years) carrying pathogenic variants in *TERT*, *RTEL1*, or *TERC* were enrolled from study 2022/07FEV/046 (Table S2). Inclusion criteria for all participants were the absence of any upper respiratory tract infection within the three weeks prior to sampling and no history of chemotherapy or radiotherapy. Written informed consent was obtained from all participants, parents, and/or legal guardians. Assent was obtained from minors when appropriate. The studies were conducted in accordance with institutional ethical guidelines.

Nasal epithelial samples were collected by a trained physician using a standardized brushing technique. A sterile cytology brush was gently inserted into the lower half of each nostril, targeting the middle turbinate and the lower part of the nasal cavity below it. The brush was rotated to obtain epithelial cells, with one brushing performed per nostril. Both brushes were immediately immersed in 1 ml DMEM/F12 medium (11039021, Gibco, Thermo Fisher Scientific) supplemented with 10% FBS (SV30160.03, Cytiva) and 1% Penicillin/Streptomycin solution (PS-B, Capricorn Scientific). Cells were mechanically dissociated from the brushes and divided into three fractions, with cell numbers adjusted according to the requirements of each downstream application. The first fraction, used for immunofluorescence combined with FISH (IF-FISH), was applied to slides using a Cytospin centrifuge ($7 \times g$, 5 min). The second fraction, intended for SA- β -Gal staining, was briefly centrifuged (30 s), the supernatant discarded, and the pellet fixed in 4% formaldehyde (F/1501/PB15, Fisher Scientific) in 1x PBS for 5 min. After a second brief spin (30 s) and supernatant removal, the pellet was resuspended in 1x PBS and applied to slides *via* Cytospin ($113 \times g$, 10 min). The third fraction, reserved for qRT-PCR analyses, was pelleted (30 s spin) and stored at -80°C .

Telomere restriction fragment (TRF) length analysis

TRF analyses were performed on genomic DNA extracted from lung tissue samples, as described previously (Episkopou et al. 2019), using 5 µg of RNase-treated genomic DNA digested with 20 U *Hinfl* (R0155S, NEB) and 20 U *RsaI* (R0167L, NEB) and a [γ -³²P] ATP (BLU502A250UC, Revvity)-labelled (TAACCC)₄ probe (Eurogentec). Smart Ladder (MW-1700-10, Eurogentec) was run together with the samples, stained with SYBR Safe DNA Gel Stain (S33102, Thermo Fisher), and imaged with a ruler. The blots were exposed to a phosphor screen, scanned on Phosphorimager (GE Healthcare), and quantified using the online available WALTER (Web-based Analyser of the Length of Telomeres) software (<https://www.ceitec.eu/chromatin-molecular-complexes/rg51/tab?tabId=125#WALTER>).

Immunofluorescence and telomeric FISH

The protocol used to detect TIF in cultured cells (Episkopou et al. 2019) was adapted. For TIF detection on frozen tissue sections, 5 µm-thick sections were briefly incubated in cold acetone for 5 min at room temperature (RT), then washed with 1x PBS before fixation in 4% formaldehyde in 1x PBS for 10 min at RT under gentle agitation. After an additional wash with 1x PBS for 5 min at RT, samples were heated in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven (450 W), incubated in permeabilization buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100) for 1 h at 37°C, and then washed three times for 3 min each in 0.1% Tween-20 in 1x PBS (PBS-T). Tissue sections were then incubated for 45 min at RT in 400 µl of blocking solution (2% normal goat serum (S26-M, Sigma-Aldrich), 0.2% BSA (A7030, Sigma-Aldrich) and 0.1% Triton X-100 in 1x PBS). Primary antibodies (Table S3) were diluted in the same blocking solution and incubated overnight at 4 °C: γ-H2AX (1:1000) and TRF2 (1:2000). After three 3-min washes at 45 °C in PBS-T, samples were incubated for 40 min at 45 °C with secondary antibodies (Table S3) diluted 1:400 in blocking solution. Samples were

then washed three times for 3 min each in PBS-T at 45 °C, followed by three final washes in 1x PBS at RT. After air drying, samples were mounted with fluorescence mounting medium (S302380-2, Agilent Technologies) containing 0.1 µg/ml DAPI (D9542, Sigma-Aldrich).

For TIF detection in nasal brushing samples, we used a combination of telomeric FISH and immunofluorescence (IF) against γ-H2AX. Nasal epithelial cells, spread on slides, were first incubated for 5 min in permeabilization buffer, then fixed for 15 min in 4% formaldehyde in 1x PBS, and subsequently re-incubated in permeabilization buffer for 10 min. IF steps were performed as described above, using antibodies against γ-H2AX (1:1000) and anti-mouse Alexa Fluor 647 as secondary antibodies (1:400) (Table S3). After washes, cells were fixed again for 2 min with 4% formaldehyde in 1x PBS, washed three times 3 min with 1x PBS, and incubated with RNase A solution (0.1 mg/ml, R4875, Sigma-Aldrich) for 1h at 37°C. Slides were washed again three times 3 min with 2x SSC before incubation in permeabilization buffer for 10 min at RT. After a last fixation for 2 min with 4% formaldehyde in 1x PBS, slides were dehydrated in successive ethanol bath (70-80-90-100%), 2 min each. Slides were air-dried before incubation with the hybridization solution (160 nM G-rich red LNA (5'TYE563/GGGT+TAGGG+T+TAG+GGTTAGGG+T+TAGGG+T+TAGGG+TTA/3'TYE563, 339414, Qiagen) telomeric probe, 50% deionized formamide (P040.2, Carl Roth), 2x SSC, 1x Blocking reagent (11096176001, Roche)). The first incubation was performed at 83°C for 3 min, then slides were cooled down to RT for 2 h. Finally, slides were washed two times for 15 min in 50% formamide, 2x SSC, 20 mM Tris-HCl pH 7.4, and then three times for 5 min in 150 mM NaCl, 0.05% Tween-20, 50 mM Tris-HCl pH 7.4. Slides were dehydrated as above and air-dried before mounting with fluorescence mounting medium.

For detection of lysosomes and CD68+ cells in nasal brushing samples, spread cells were permeabilized for 30 min at RT in Tris-HCl buffer with 0.5% Triton X-100, rinsed with 1x PBS, and blocked for 1 h

at RT in 1x PBS containing 0.1% Triton X-100, 0.21% BSA, and 2.1% normal goat serum. Slides were then incubated overnight at 4°C with primary antibodies (Table S3) diluted in blocking solution: LAMP2 (1:200), β -catenin (1:200) or CD68 (1:200). Samples were incubated three times for 3 min each in PBS-T at 45°C before incubation for 40 min at RT with secondary antibodies diluted in blocking solution: anti-mouse Alexa Fluor 647 and anti-rabbit Alexa Fluor 488 (1:1000). This was followed by another series of three 3-min washes with PBS-T at 45 °C, and a final series of three 3-min washes with 1x PBS at RT. Slides were mounted as described above. Images were acquired using a Cell Observer Spinning Disc confocal microscope (Zeiss) with either a 40x or a 100x oil immersion objective. Image processing was performed using ImageJ (imagej.nih.gov).

p16 immunohistochemistry on lung sections

p16 immunochemistry (IHC) was performed on 5 μ m-thick FFPE lung sections according to the manufacturer's instructions using the mouse E6H4 anti-p16 monoclonal antibody and the VENTANA detection kit (705-4793, Roche) on a BenchMark ULTRA automated slide stainer. Image acquisition and processing was performed as described above. After IHC, IPF lung sections were stained with Alcian Blue (A3157-10G, Sigma-Aldrich) to detect fibroblastic foci.

RNA extraction and qRT-PCR

For lung samples, total RNA was extracted from ten 10 μ m-thick cryosections of frozen tissue using TriPure reagent (11667165001, Roche), following the protocol described previously (Episkopou et al. 2019). For nasal brushing samples, total RNA was extracted from cell pellets using the RNeasy® Plus kit (74006, Qiagen). Pellets were resuspended in 350 μ l RLT buffer containing 1% β -mercaptoethanol, followed by the addition of 350 μ l 70% ethanol. The suspension was transferred to the spin column and centrifuged for 30 s at 8000 x *g* at RT. Bound RNA was washed with 350 μ l RW1 buffer and centrifuged for 30 s at 8000 x *g*. On-column DNase digestion (2.7 U/ μ l; 79256,

Qiagen) was performed to remove genomic DNA, followed by an additional RW1 wash. Two washes with 500 μ l RPE buffer were carried out, each followed by centrifugation (30 s at 8000 x *g*, then 2 min at 8000 x *g*), and a final 1 min centrifugation at maximum speed. RNA was eluted by incubating 25 μ l RNase-free water (RF) on the membrane for 20 min, followed by centrifugation for 30 s at 8000 x *g*. cDNA synthesis and qRT-PCR analyses were performed as described previously (Episkopou et al. 2019) using primers described in Table S4.

SA- β -Gal activity

For lung samples, the SA- β -Gal activity assay was performed at pH 5.6 on 16 μ m-thick sections as described previously (Froidure et al. 2020). Briefly, after fixation with 0.2% formaldehyde for 10 min, sections were rinsed with 1x PBS before overnight incubation at 37°C in staining solution (40 mM citric acid/Na phosphate, pH 5.6, 5 mM $[K_4[Fe(CN)_6] \cdot 3H_2O]$ (P9387, Sigma-Aldrich), 5 mM $K_3[Fe(CN)_6]$ (702587, Sigma-Aldrich), 150 mM NaCl, 2 mM $MgCl_2$, 1 mg/ml X-gal (B4252, Sigma-Aldrich)). The next day, slides were scanned using the Mirax Midi Imaging system (Zeiss). Images were analyzed with QuPath (qupath.github.io). For nasal brushing samples, spread cells were first rinsed with 1x PBS before incubation for 18 h at 37°C in the staining solution at pH 6. A positive control was performed at pH 4. After rinsing with 1x PBS and mounting with 90% glycerol in distilled water, images were acquired using an EVOS XL Core microscope (Invitrogen, Thermo Fisher Scientific) in brightfield mode to count blue-stained cells and in phase-contrast mode to count total cells, using a 40x objective. Results at pH 6 were excluded if the percentage of blue cells at pH 4 was below 85%.

Single Telomere Length Analysis (STELA)

STELA analysis was performed as described previously (Baird et al. 2003). Briefly, genomic DNA was extracted using QIAmp DNA Micro

Kit (56304, Qiagen) and eluted in 35 µl 10 mM Tris–HCl (pH 8) and 1 µl of Telorette2 linker (10 µM) added to the DNA solution. This was diluted to 500 pg/µl. One µl of this diluted DNA/Telorette2 solution were subjected to PCR in a 10 µl reaction containing 0.5 µM 17p telomere-specific primer (17pseq1rev: 5'- GAATCCACGGATTGCTTTGTGTAC-3'), 0.5 µM Teltail primer and 0.5 U of a 10:1 mixture of Taq (Thermo Fisher Scientific) and Pwo polymerase (11644955001, Roche). PCR amplicons were resolved by 0.5% TAE agarose gel electrophoresis and were detected by Southern hybridization with a random-primed α-³³P-labelled (Hartmann Analytic GmbH) TTAGGG repeat probe together with a probe to detect 1kb (Stratagene) and 2.5kb (170-8205, BioRad) molecular weight markers. The blots were exposed to a phosphor screen, scanned on Typhoon FLA 9500 phosphorimager (GE Healthcare) and analysed using ImageQuant TL (GE Healthcare).

Bulk RNA sequencing and deconvolution analysis

RNA sequencing was performed on five nasal brushing RNA samples, which were processed by MacroGen Inc.. Libraries were prepared using the TruSeq Stranded Total RNA kit with Ribo-Zero H/M/R ribodepletion protocol, targeting removal of ribosomal RNA. Sequencing was carried out on the Illumina NovaSeq platform under the Transcriptome Resequencing application. Data were generated in paired-end mode with a read length of 150 bp, yielding approximately 60 million raw reads per sample (30 million paired-end reads, PE150). Fastq files were processed using a standard RNA-seq pipeline including Trimmomatic (v0.39) (Bolger et al. 2014) to remove low quality reads, HISAT2 (v2.2.1) (Kim et al. 2015) to align reads to the human genome (GRCh38), and Homo_sapiens.GRCh38.105.chr.gtf. and featureCounts from Subread (v2.0.3) (Liao et al. 2014) to estimate gene expression levels. Deconvolution analysis was performed with DeconRNASeq R Bioconductor package (Gong and Szustakowski 2013) using a reference expression matrix, derived from published scRNA-seq data

(Deprez et al. 2020), in which 4842 nasal brushing cells from patient D353 were used.

Olfactory tests

Healthy volunteers (n = 72, 42 females and 30 males, 18-97 years) were recruited from two independent clinical studies, all approved by the local ethics committee (Comité d’Ethique Hospitalo-Facultaire Saint-Luc–UCLouvain) under the following references: 2016/21MAR/121 and 2022/07FEV/046 (Table S2). Individuals with TBD (n = 10, 6 females and 4 males, 18-62 years) carrying pathogenic variants in *TERT*, *RTEL1*, or *TERC* were enrolled from study 2022/07FEV/046 (Table S2). Written informed consent was obtained from all participants, parents, and/or legal guardians. Assent was obtained from minors when appropriate. The studies were conducted in accordance with institutional ethical guidelines.

Olfactory abilities were measured with the Sniffin’ Sticks® test (200112, MediSense) (Hummel et al. 2017). The test yields three separate scores: odor threshold (T), discrimination (D), and identification (I). The stimuli are presented *via* felt-tip pens filled with odor solutions, which are held just below the nostrils and gently moved past them for several seconds. The threshold task involves phenylethanol, serially diluted across sixteen concentrations. In each trial, participants are given three pens, only one of which contains the odor. Using a forced-choice procedure, they attempt to detect the correct pen. A staircase rule was applied, where performance determined whether weaker or stronger concentrations were presented. After seven reversals, the threshold value was computed as the mean of the last four reversals, producing a score between 1 and 16. Discrimination is assessed with sixteen sets of three pens, where two pens contain the same odor and the third differs. With eyes closed, participants indicate the odd stimulus. The number of correct responses provides the discrimination score (0-16). For identification, sixteen familiar odors – commonly recognized by most healthy

individuals – are presented individually. Participants choose the correct label from four options read aloud by the examiner, yielding an identification score between 0 and 16. The overall measure of olfactory performance, the TDI score, is obtained by summing the three subscores, with a maximum of 48 points.

Statistical analyses

All analyses were performed using the GraphPad Prism software. Graphs were also obtained with GraphPad Prism. The statistical tests that were applied are detailed in the figure legends.

RESULTS

1. TIF increase occurs late in the aging human lung epithelium

To monitor TIF formation in the aging human lung, we used tissue samples from organ donors aged 16 to 88 (**Table S1**). Consistent with previous findings (Lee et al. 2021; Van Slambrouck et al. 2025), we observed a linear, age-related decrease in telomere length (TL) (**Fig. 1A-B**). In our lung tissue cohort, the age-dependent TL attrition was of about 42 ± 5 bp per year, in line with the 20-60 bp range previously proposed for yearly rates of TL reduction in human tissues (Takubo et al. 2010).

TIF are identified by the co-localization of DNA damage markers with telomeres (d'Adda di Fagagna et al. 2003). Here, TIF were assessed by immunofluorescence-based co-detection of telomeres (TRF2) and γ -H2AX DNA damage marker (**Fig. 1C and S1A**). In the lung epithelium, the average number of TIF per nucleus was significantly higher in tissues from donors aged 75 to 88 compared to those aged 16 to 47, with a median of 3.5 TIF per nucleus in the oldest group (**Fig. 1D**). In contrast, this increase was not significant in non-epithelial regions (stroma) of the same lung tissues (**Fig. 1C and 1E**). In the oldest lung epithelial tissues, nuclei frequently contained more than one TIF (**Fig. 1F**). In line with the elevated TIF levels in the aging lung epithelium, p16 staining was notably more intense in this region (**Fig. 1G**). Contrasting with previous work however (Lee et al. 2021), our qRT-PCR analysis of *p16* mRNA in total lung tissue extracts did not reveal any significant increase with aging, although a tendency was observed (**Fig. S1B-C**).

Collectively, these findings indicate that the rise in TIF frequency occurs only at a very advanced age, specifically within the lung epithelium of donors aged 75 and older.

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

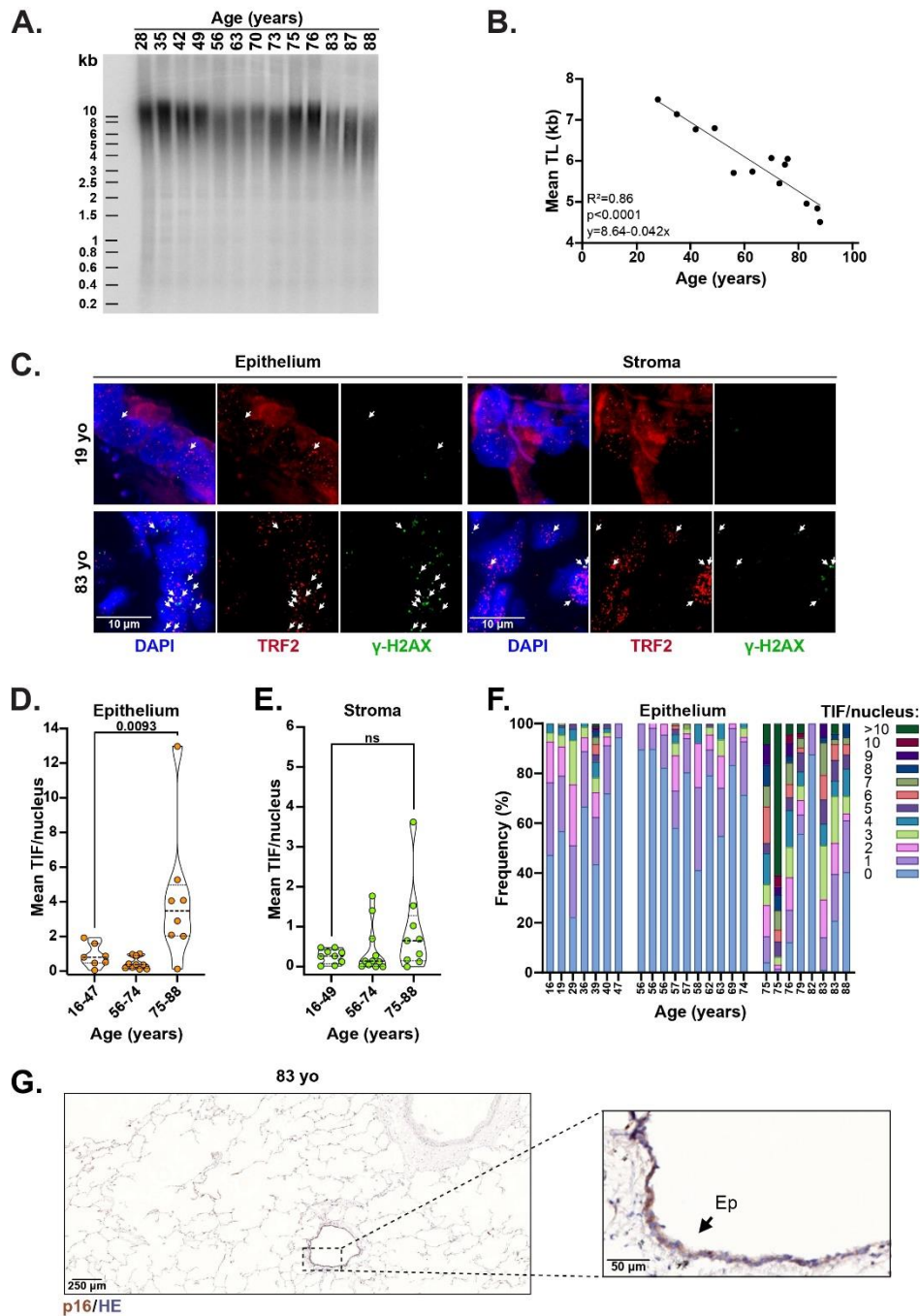


Figure 1. TIF increase occurs late in the aging human lung epithelium. **A.** TRF analysis of TL in lung tissues from control donors (Ctrl) aged 28 to 88 years. Ladder (kb). **B.** Linear

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regression analysis corresponding to panel A. **C.** Representative images of TIF analysis in the epithelium (left) and stroma (right) of control lungs from 19- and 83-year-old donors. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bar: 10 μ m. **D.** Mean number of TIF per nucleus in the lung epithelium of donors across the specified age ranges. On average, around 75 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Mann-Whitney test. **E.** As in panel D, but for the lung stroma. On average, around 100 nuclei were analyzed per sample. Mann-Whitney test. **F.** Distribution of nuclei by the number of TIF per nucleus in the lung epithelium of donors at the indicated ages. **G.** Representative images of p16 staining by immunohistochemistry in lung sample from a 83 years old donor. HE: Hematoxylin-Eosin. Ep: epithelium. Bar scale: 50 μ m

2. Premature TIF and senescence in IPF lung epithelium

Our observation that TIF frequency rises significantly only at very advanced ages in the human lung epithelium may help account for the more than 50-fold increase in IPF prevalence among individuals aged 75 and older compared to those in their 20s and 30s (Schneider et al. 2021). To explore whether age-related TIF contributes to increased susceptibility to IPF in older adults, we hypothesized that if TIF formation plays a causal role in IPF pathogenesis—rather than being a consequence of the disease—then TIF frequency in IPF lungs may approximate that seen in the oldest control lungs. To address this, we analyzed TIF in lung samples from IPF patients aged 53 to 66, age-matched controls (aged 56 to 63), and older controls aged 75 and above (**Table S1**). TIF abundance in the epithelial tissue of IPF lungs (median of 2.2 TIF/nucleus) was approximately fivefold higher than in age-matched controls (median of 0.4 TIF/nucleus) (**Fig. 2A-B and S2A**). Consistent with our hypothesis, this level was comparable to that observed in the lung epithelium of control lungs from donors aged 75 and above (**Fig. 2A-B and S2A**). Similar results were obtained when comparing the percentage of cells containing at least two TIF per nucleus (**Fig. 2C**). In contrast, differences in TIF abundance among non-epithelial cells from the stroma were not statistically significant (**Fig. 2D-E**).

The “second hit” hypothesis posits that external insults to an already compromised, aging lung epithelium contribute to the onset of IPF

(Hewitt et al. 2025). Our findings indicate that TIF may be involved in establishing this age-related epithelial susceptibility. However, since TIF levels were comparable between lungs from IPF donors and age-matched controls (≥ 75 years) with markedly different degrees of cellular senescence, TIF alone appear insufficient to drive the extensive senescence observed in IPF. This is supported by significantly elevated *p16* mRNA levels in IPF lungs (**Fig. 2F**), and strong p16 protein staining in epithelial cells near fibrotic regions, marked by Alcian blue, consistent with previous studies (Minagawa et al. 2011; Schafer et al. 2017) (**Fig. 2G and S2B**). The heightened senescence observed in IPF is further supported by a concurrent increase in SA- β -Gal activity within the epithelial layer (**Fig. 2H**).

Collectively, these findings support a potential causal role for TIF in IPF pathogenesis and reinforce the hypothesis that additional external insults are required to initiate the pronounced cellular senescence observed in the IPF lung epithelium, characterized by elevated p16 expression and SA- β -Gal activity.

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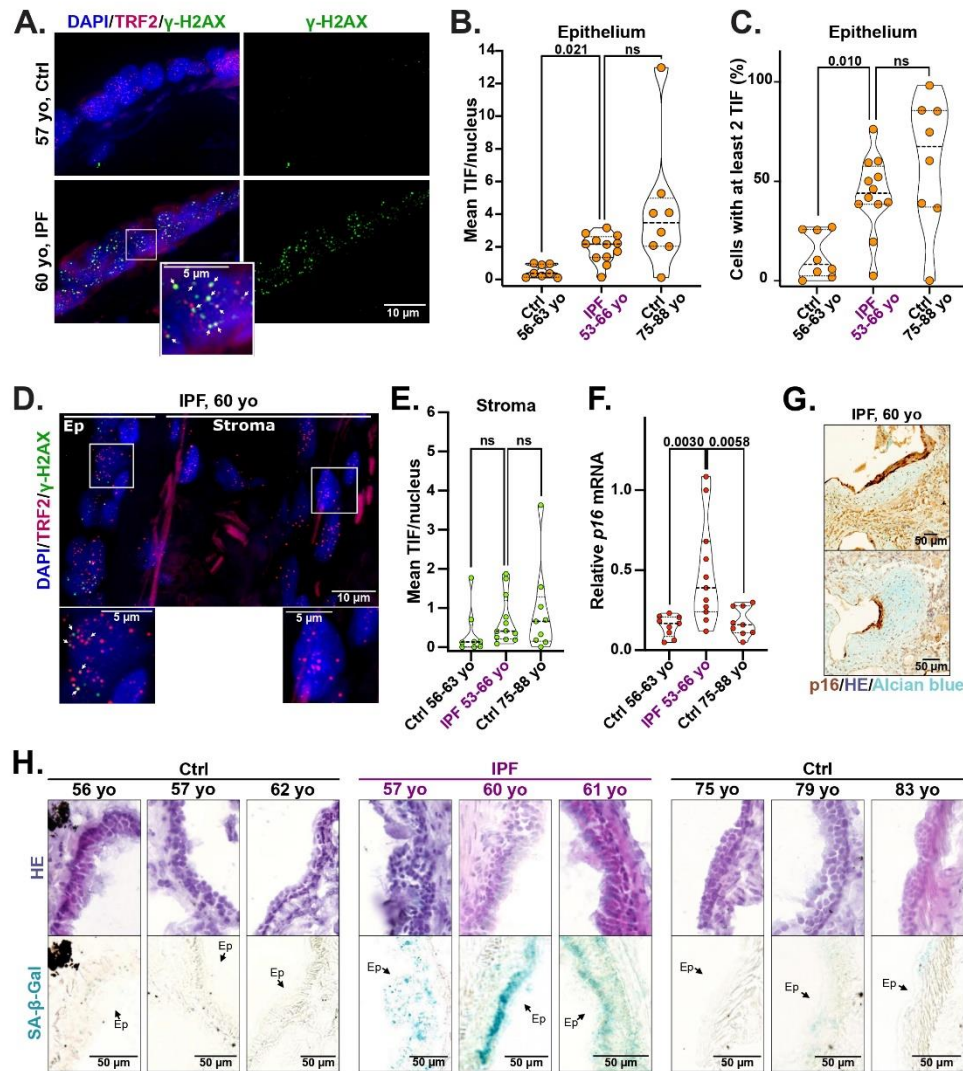


Figure 2. Premature TIF and senescence in IPF lung epithelium. **A.** Representative images of TIF analysis in the lung epithelium from a 57-year-old control donor (Ctrl) and a 60-year-old donor with IPF. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bars: 5 and 10 μ m. **B.** Mean number of TIF per nucleus in the epithelium of Ctrl or IPF lungs across the specified age ranges as indicated on the graph. On average, around 90 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal-Wallis test. ns: not significant. **C.** Percentage of nuclei showing at least 2 TIF in the epithelium of Ctrl or IPF lungs across the specified age ranges as indicated on the graph. Violin plots showing median, first and third quartile. Ordinary one-way ANOVA test. **D.** As in panel A but including both epithelial (Ep) and stromal regions of the IPF lung. Bar scales: 5 and 10 μ m. **E.** Mean number of TIF per nucleus

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in the stromal region of Ctrl or IPF lungs across the specified age ranges as indicated on the graph. On average, around 100 nuclei were analyzed per sample. Kruskal-Wallis test. ns: not significant. F. qRT-PCR analysis of p16 expression in lung tissue samples from age-matched Ctrl and IPF donors, as well as Ctrl donors aged 75 and older. p16 mRNA levels were normalized to ACTB mRNA and to one IPF lung sample used as control. Ordinary one-way ANOVA test. G. p16 staining by immunohistochemistry and Alcian blue staining of fibrotic area in lung sample from a 60-year-old donor with IPF. HE: Hematoxylin-Eosin. Bar scale: 50 μ m. H. Representative images of SA- β -Gal activity staining in Ctrl or IPF lung samples from donors at the indicated age. Ep: epithelium. Hematoxylin-Eosin (HE) staining is shown above. Bar scale: 50 μ m

3. Telomere shortening without major TIF induction in aging nasal epithelial cells

Cellular aging progresses along distinct, organ-specific trajectories and exhibits temporal variability (Schaum et al. 2020). To investigate whether senescence markers in the upper respiratory tract mirror those observed in the aging lung epithelium, we developed a minimally invasive nasal brushing method to collect epithelial cells from healthy donors ranging in age from 2 to 97 years (**Table S2 and Fig. S3**). First, we assessed whether TL decreases with age in nasal epithelial cells. Using STELA analysis (Baird et al. 2003) on samples from young (n=3) and older (n=5) donors, we estimated an age-related TL attrition rate of approximately 64 ± 8 base pairs per year (**Fig. 3A-B**, black). This value is comparable to the 59–60 bp/year telomere shortening rate observed in the colon and the esophagus, with high regenerative activity (Takubo et al. 2010). Nasal brushing samples from two individuals with telomere biology disorders (TBD), carrying mutations in either the *TERC* (age 56) or *TERT* (age 29) subunit of telomerase, revealed markedly short telomeres, comparable to the average TL observed in nasal epithelial cells from control individuals aged 75 and older (**Fig. 3A-B**). Collectively, these findings support the utility of nasal epithelial cells as a valid model for studying telomere dynamics during aging.

Analysis of TIF formation in nasal epithelial cells revealed a modest age-associated increase in TIF levels (**Fig. 3C-D**). Despite this trend, the median number of TIF per nucleus remained very low, rising only slightly from 0 to 0.04 across age groups (**Fig. 3D**), indicating a

generally low TIF abundance in this tissue. Nasal epithelial cells from TBD patients aged 19 to 62 displayed a higher TIF burden compared to age-matched controls, but levels were similar to those seen in the older control group aged 80 and above (**Fig. 3E-G**). This supports the notion of overall low TIF abundance in nasal epithelium. Additionally, our finding that the average number of γ -H2AX foci in samples from older individuals did not significantly increase (**Fig. S4**) argues against the possibility of a bias in TIF detection resulting from telomeres being too short to be visualized by FISH. In the olfactory neuroepithelium – located near the nasal epithelium – and sampled from two deceased donors aged 83 and 94 years, TIF levels were also extremely low (median of 0) (**Fig. 3H-I**). These results contrast with the increased TIF abundance observed in the aging lung epithelium, highlighting the previously reported variability in age-dependent TIF (Jeyapalan et al. 2007).

These findings indicate that, although TL decreases with age, nasal epithelial cells from healthy individuals show minimal accumulation of TIF.

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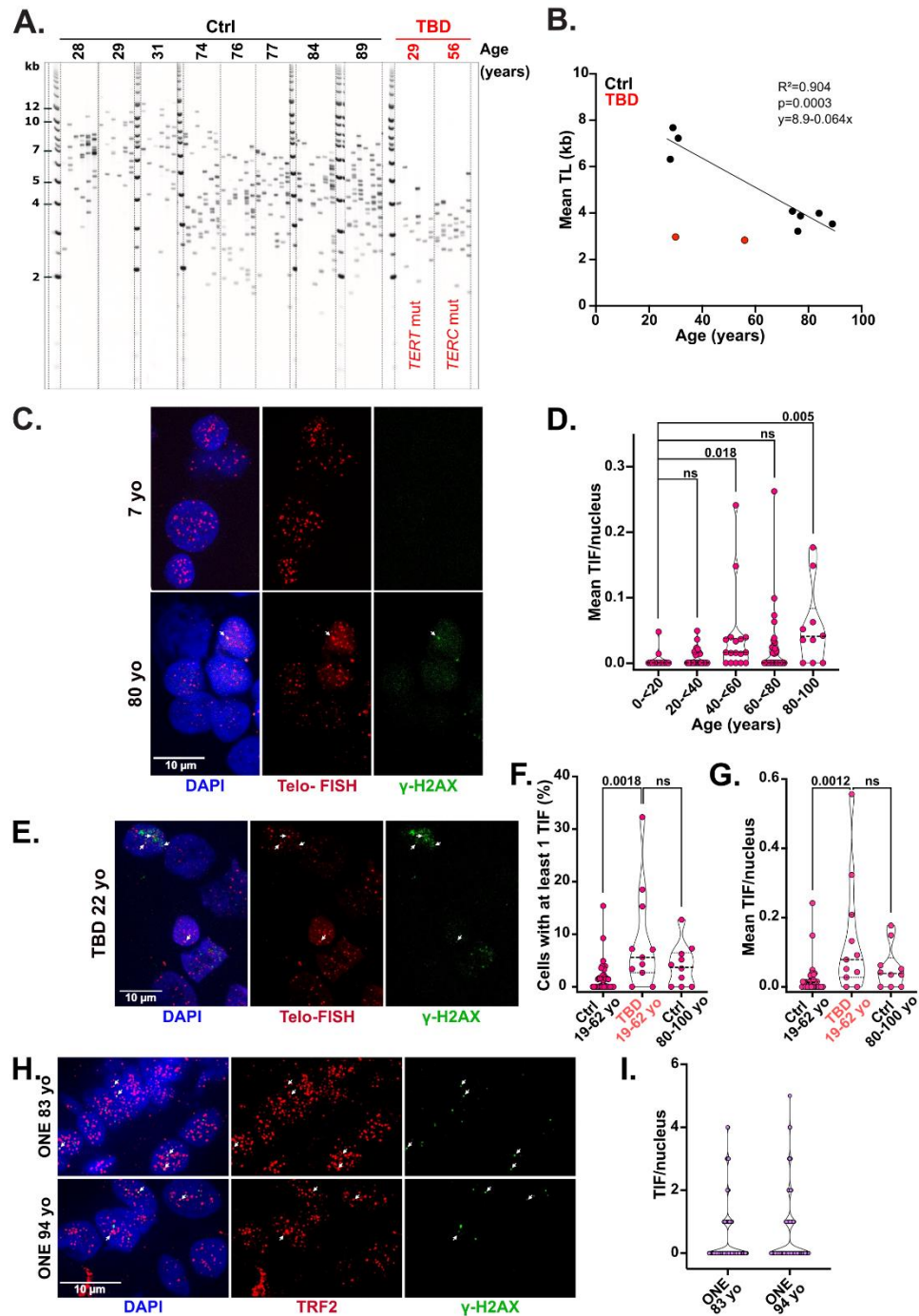


Figure 3. Telomere shortening without major TIF induction in aging nasal epithelial cells. A. STELA analysis of TL in nasal brushing samples from donors without (Ctrl) or with

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telomere biology disorder (TBD). The age of the donors is indicated. Ladder (kb). B. Linear regression analysis corresponding to panel A using nasal brushing samples from the Ctrl donors (black dots). Red dots represent TBD donors. C. Representative images of TIF analysis in nasal brushing samples from Ctrl donors aged 7 and 80 years. Telomeres are detected by FISH with a telomeric probe (Telo-FISH, red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bar: 10 μ m. D. Mean number of TIF per nucleus in the nasal brushing samples of Ctrl donors across the specified age ranges. On average, around 65 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal-Wallis tests. ns: not significant. E. As in panel C, but in the nasal brushing sample from a 22-year-old donor with TBD. Arrows indicate TIF. Scale bar: 10 μ m. F. Percentage of nuclei showing at least one TIF per nucleus based on the analysis described in panel F. Violin plots showing median, first and third quartile. Kruskal-Wallis tests. ns: not significant. G. Mean number of TIF per nucleus in the nasal brushing samples from Ctrl and TBD donors as indicated on the graph. On average, around 80 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal-Wallis tests. ns: not significant. H. Representative images of TIF analysis in the ONE tissue of two donors aged 83 and 94 years. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bar: 10 μ m. I. Number of TIF per nucleus in the ONE samples from panel H. A total of 85 and 101 nuclei were counted per sample, respectively

4. Early proteostasis disruption in nasal epithelial cells

As no single marker can definitively identify senescent cells (Gorgoulis et al. 2019), and given the minimal accumulation of TIF observed in aged nasal epithelial cells, we proceeded to examine additional senescence markers in the nasal brushing samples.

The colorimetric assay for SA- β -Gal activity (Dimri et al. 1995), which reflects the elevated lysosomal content characteristic of senescent cells undergoing proteotoxic stress, remains the most widely used approach for identifying senescent cells both *in vitro* and *in vivo* (Dimri and Dimri 2024). Using this method, we found that the proportion of SA- β -Gal⁺ cells increases with age, showing a significant rise in individuals aged 40 and above, followed by a plateau around the age of 60 (**Fig. 4A**). In the oldest age group – individuals over 80 years – approximately 30% of the collected cells were SA- β -Gal⁺ (**Fig. 4A**). Notably, this increase in SA- β -Gal positivity occurred well before the modest rise in TIF frequency observed in the oldest individuals (**Fig.**

4A), indicating that SA- β -Gal expression does not result from TIF induction.

One limitation of using SA- β -Gal as a senescence marker is its tendency to also stain macrophages (Sharpless and Sherr 2015). To rule out the possibility that the observed increase in SA- β -Gal positivity in nasal brushing samples was due to macrophages, we measured *CD68* mRNA levels, a recognized macrophage marker. Despite the significant elevation in SA- β -Gal⁺ cells, there was no statistically significant difference in *CD68* mRNA expression between the 0-<40 and 40-100 years age groups (**Fig. 4B**). This was confirmed by staining two independent nasal brushing samples, which revealed SA- β -Gal positivity clearly distinct from the proportion of CD68-positive cells (**Fig. 4C**). Alternatively, age-related shifts in the cellular composition of nasal brushings could potentially confound interpretations of their association with aging. To investigate this possibility, we conducted bulk RNA-seq on nasal brushing samples from three younger donors (aged 7.5, 11.5, and 29.5) and two older donors (aged 74 and 77). Deconvolution analysis of the transcriptomic data revealed no substantial age-related differences in cell type composition that may account for the observed changes in SA- β -Gal positivity (**Fig. 4D**).

Earlier studies proposed that elevated SA- β -Gal activity in senescent fibroblasts reflects increased lysosomal content and accumulation of degradative autolysosomes (Gerland et al. 2003). Accordingly, the rise in SA- β -Gal positivity in nasal epithelial cells around age 40 may indicate an early impairment in proteostasis. To strengthen this observation, we employed an orthogonal method by staining samples with an antibody against LAMP2 lysosomal-associated membrane protein to assess lysosome enlargement. Cells were considered LAMP2⁺ if they showed intense cytoplasmic staining (**Fig. 4E**). Consistent with SA- β -Gal staining and prior findings of increased LAMP2 expression in senescent melanoma cells (Rovira et al. 2022), the proportion of LAMP2⁺ nasal epithelial cells rose significantly with

donor age and showed a strong correlation with SA- β -Gal positivity (**Fig. 4F**).

Finally, we examined whether the increased SA- β -Gal positivity in aging nasal epithelial cells was linked to elevated expression of the senescence markers *p21* or *p16*. While *p21* mRNA showed a moderate correlation with SA- β -Gal positivity (**Fig. 4G**), *p16* expression levels did not correlate with SA- β -Gal positivity in nasal epithelial cells (**Fig. 4H**).

Together, these results point to an association between aging of nasal epithelial cells and proteostatic stress.

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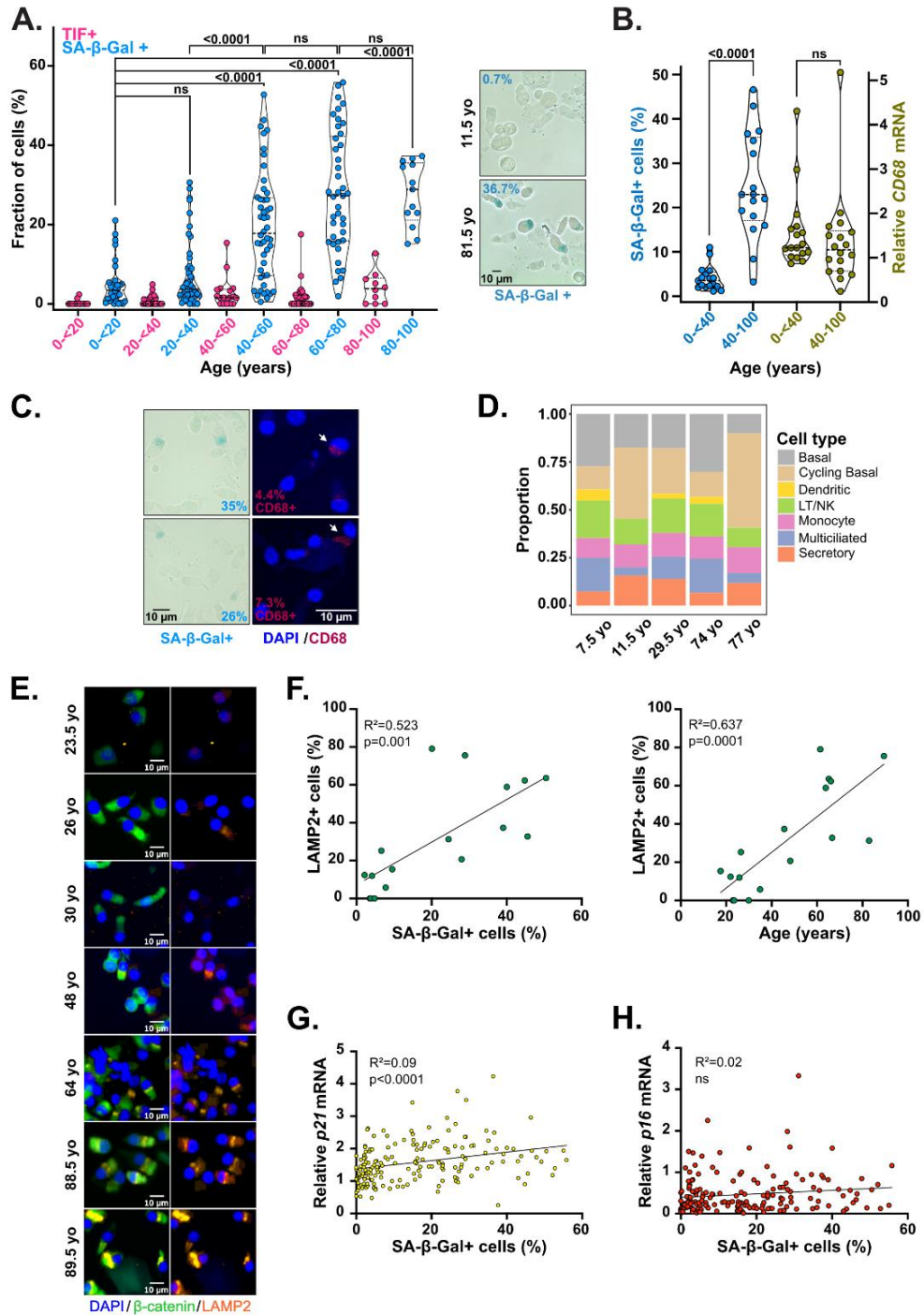


Figure 4. Early proteostasis disruption in nasal epithelial cells. **A.** Percentage of SA- β -Gal-positive cells (SA- β -Gal+, blue) in nasal brushing samples and percentage of nuclei showing at least one TIF per nucleus (TIF+, pink) across the specified age ranges. For SA- β -Gal staining, on average, around 500 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal-Wallis tests. ns: not significant. Right: representative images of SA- β -Gal staining from two donors aged 11.5 and 81.5 years. **B.** Percentage of SA- β -Gal-positive cells (blue) and relative CD68 mRNA expression (khaki) in a subset of nasal brushing samples from donors below or above 40 years old. CD68 mRNA levels were normalized to ACTB mRNA and to a reference sample. Mann-Whitney tests. ns: not significant. **C.** Left: representative images of SA- β -Gal staining in two nasal brushing samples with the indicated percentages of SA- β -Gal+ cells. Right: CD68 detection by immunofluorescence (red) on the same nasal brushing samples. DNA is stained with DAPI (blue). The percentage of CD68-positive cells (arrows) is indicated. Scale bar: 10 μ m. **D.** Estimated proportions of major cell types obtained by deconvolution analysis of nasal brushing RNA-seq samples. **E.** Representative images of LAMP2 staining (orange) in nasal brushing samples from donors at the indicated ages. Cytoplasm is detected by β -catenin staining (green). DNA is stained with DAPI (blue). Scale bar: 10 μ m. **F.** Linear regression analyses showing the percentage of LAMP2+ cells plotted against either SA- β -Gal+ cells (left) or donor age (right). **G-H.** Linear regression analyses showing p21 (**G**) or p16 (**H**) expression levels in nasal brushing samples plotted against the percentage of SA- β -Gal+ cells. ns: not significant.

5. SA- β -Gal activity in nasal epithelial cells negatively correlates with olfactory function

Olfactory function gradually declines beginning in early adulthood, with the most marked reduction observed in odor threshold sensitivity, as opposed to odor discrimination or identification (Oleszkiewicz et al. 2019). Odor discrimination and identification rely on both the olfactory nasal epithelium and central brain regions such as the entorhinal cortex and hippocampus to process the information (Kjelvik et al. 2012). Conversely, odor threshold is more directly influenced by olfactory nasal epithelium function (Hummel et al. 2017). To investigate whether aging of the olfactory nasal epithelium contributes to olfactory decline, we assessed odor threshold in healthy individuals aged 18 to 97. Using the Sniffin' Sticks test, we found that declining threshold scores with age were associated with increased SA- β -Gal positivity in nasal epithelial cells (**Fig. 5A-B**), suggesting a potential link between proteostasis loss in these aging cells and olfactory decline. Since the olfactory epithelium supports the maintenance and function of olfactory neurons, its aging is also likely to impair overall

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olfactory performance. In line with this, the total TDI score showed a negative correlation with SA- β -Gal positivity in nasal epithelial cells (**Fig. 5C**). Interestingly, TBD patients did not exhibit any premature olfactory deficit (**Fig. 5D**), further supporting the idea that telomere dysfunction is not a driver of aging in the olfactory nasal epithelium.

Taken together, these findings indicate that impaired proteostasis in the aging olfactory nasal epithelium contributes to the age-related decline in olfactory function.

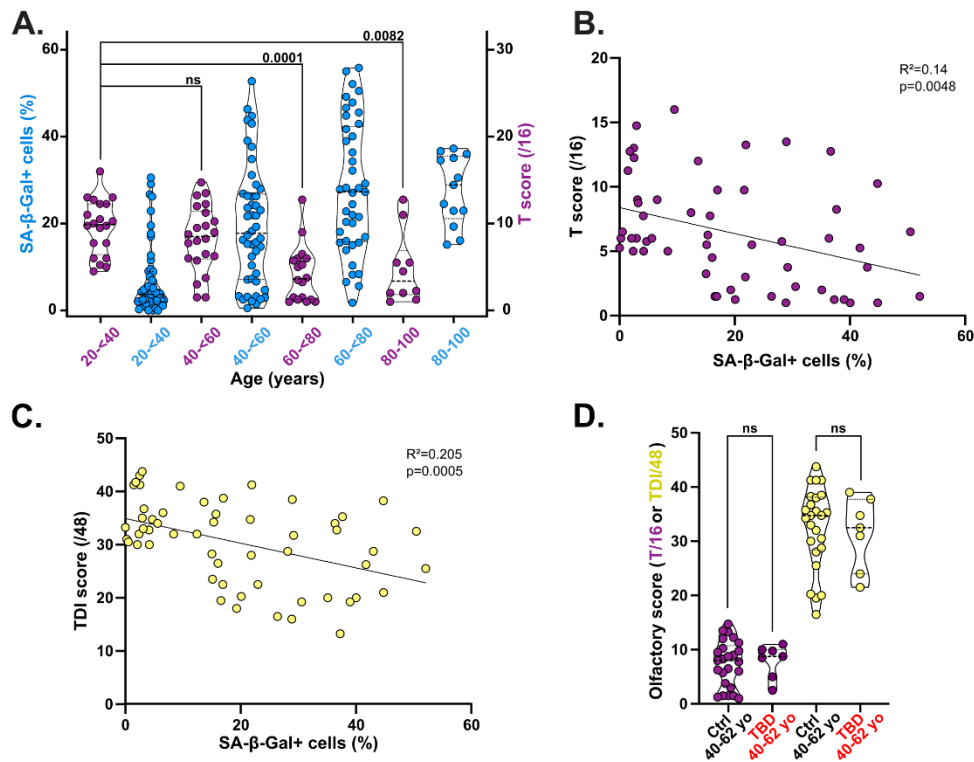


Figure 5. SA- β -Gal activity in nasal epithelial cells negatively correlates with olfactory function. **A.** Percentage of SA- β -Gal+ cells (blue, same as Fig. 4A) in nasal brushing samples and Threshold (T) olfactory score (mauve) in donors across the specified age ranges. Violin plots showing median, first and third quartile. Kruskal-Wallis tests. ns: not significant. **B.** Linear regression analysis of T score relative to the percentage of SA- β -Gal+ nasal brushing cells in a total of 75 donors aged 18 to 97 years. **C.** As in panel B, but for the TDI (Threshold + Discrimination + Identification) olfactory score. **D.** T (mauve) or TDI (yellow) olfactory scores

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in age-matched Ctrl and TBD patients. Violin plots showing median, first and third quartile. Unpaired t tests. ns: not significant.

DISCUSSION

In patients with TBD, telomere dysfunction is a well-established and widely accepted contributor to accelerated cellular and tissue aging, as well as the early onset of age-related diseases (Revy et al. 2023; Rossiello et al. 2022). In contrast, during normal aging, only low levels of damaged telomeres have been reported in various tissues of aging baboons, including skin, hippocampal neurons, and liver hepatocytes (Fumagalli et al. 2012; Herbig et al. 2006; Jeyapalan et al. 2007), as well as in skin biopsies from elderly human donors (Vitorelli et al. 2019). In both baboon and human tissues, the average TIF burden per nucleus remains modest compared to the elevated levels observed during replicative senescence *in vitro* (Jeyapalan et al. 2007; Vitorelli et al. 2019). While early studies in telomerase-deficient mice indicated that just one or a few critically short telomeres could be sufficient to trigger cellular senescence *in vivo* (Hemann et al. 2001), the extent to which TIF contribute to physiological aging in primates remains unresolved. Given the heterogeneity of cellular senescence (Cohn et al. 2023), it is also likely that the contribution of TIF to senescence is not universal across cell types or tissues.

Patients with TBD exhibit a strong predisposition to early-onset IPF, supporting a causal link between telomere dysfunction in the lung epithelium and IPF pathogenesis (Revy et al. 2023; Rossiello et al. 2022). In contrast, among individuals without TBD, IPF prevalence rises sharply only after the age of 75 (Hewitt et al. 2025; Schneider et al. 2021). Whether this is associated with TIF accumulation in the lung epithelium remains unclear, as most studies to date have focused on lung tissues from individuals younger than 80 years old. In this study, we show that TIF frequency in the lung epithelium rises significantly in donors aged 75 and older, reaching levels comparable to those observed in IPF lungs. These findings point to a potential causal link between TIF accumulation and the development of IPF during

physiological aging. However, TIF accumulation is unlikely to be sufficient for IPF induction as both SA- β -Gal activity and p16 expression levels in IPF lungs are markedly higher than those observed in the lung epithelium of even our oldest donors. This supports the "second hit" hypothesis, which proposes that IPF arises from additional exogenous insults acting on an already vulnerable, aging lung epithelium (Hewitt et al. 2025). It is therefore plausible that TIF induction contributes to this vulnerability, and that further environmental exposures, such as cigarette smoke, intensify cellular senescence and drive fibrosis. Consistent with our previous findings showing that short telomeres elevate the risk of severe COVID-19 (Froidure et al. 2020), the late-life increase in TIF within the lung epithelium may also possibly contribute to the heightened vulnerability to COVID-19-related mortality, which is reported to be approximately 50 times higher in individuals aged 80 and above compared to those aged 18-39 (Williamson et al. 2020).

Age-related alterations in the nasal epithelium have been proposed to contribute to olfactory decline (Doty and Kamath 2014; Kondo et al. 2020; Mobley et al. 2014). Although the decline in odor identification function may be partly attributed to broader age-related deterioration in central nervous system functions, which we did not address in this study, severe but transient damage to the olfactory epithelium, independent of injury to the central nervous system, has been linked to anosmia, as observed during SARS-CoV-2 infection (Bryche et al. 2020). Similarly, cumulative environmental insults to the olfactory epithelium have been proposed as a contributing factor to age-related olfactory decline (Attems et al. 2015). While the exact mechanisms by which epithelial damage contributes to age-associated olfactory loss remain unclear, they may involve functional impairments such as reduced ciliary beat frequency and prolonged nasal mucociliary clearance, both of which have been reported in individuals over the age of 40 (Ho et al. 2001). Aging of the upper respiratory tract however has been relatively understudied, and whether TIF play any role in this part of the respiratory tract remains unexplored. Our findings suggest

that TIF are unlikely to be the primary drivers of age-related senescence in nasal epithelial cells. Instead, we propose that this nasal epithelium senescence results from a gradual loss of proteostasis beginning after the age of 40. This is consistent with recent findings suggesting a widespread breakdown of proteostasis in aging human tissues, with a notable acceleration in aging processes typically occurring around the age of 50 (Ding et al. 2025). Proteostasis, the maintenance of a balanced system for protein synthesis, folding, trafficking, and degradation, can be disrupted under conditions such as oxidative stress (Korovila et al. 2017). Due to their direct exposure to the external environment, nasal epithelial cells are particularly vulnerable to oxidative stress (Yamamoto et al. 2023), which may lead to proteostatic imbalance. Recently, widespread amyloid protein aggregation was identified as a hallmark of aging across multiple human organs (Ding et al. 2025); however, whether similar protein aggregation occurs in the nasal epithelium remains to be determined. In support of this possibility, pathological accumulation of amyloid- β and PHF-tau has been reported in the olfactory epithelium of aged individuals, although the absence of tissue from younger donors in that study limits direct comparison (Arnold et al. 2010). Notably, hallmarks of aging in the nasal epithelium appeared distinct from those observed in the lung as, consistent with earlier reports (Minagawa et al. 2011), we did not detect a consistent age-related increase in SA- β -Gal staining in the epithelium of aging lungs.

Overall, our findings suggest that TIF are unlikely to serve as universal drivers of cellular senescence, which appears to arise through diverse mechanisms across different human cell types (Cohn et al. 2023). Future research involving human tissues, biopsies, or *ex vivo* cellular analyses will be essential to more accurately elucidate the mechanisms of cellular senescence in the human body, as traditional *in vitro* models of replicative senescence do not fully capture the complexity of senescence as it occurs *in vivo*.

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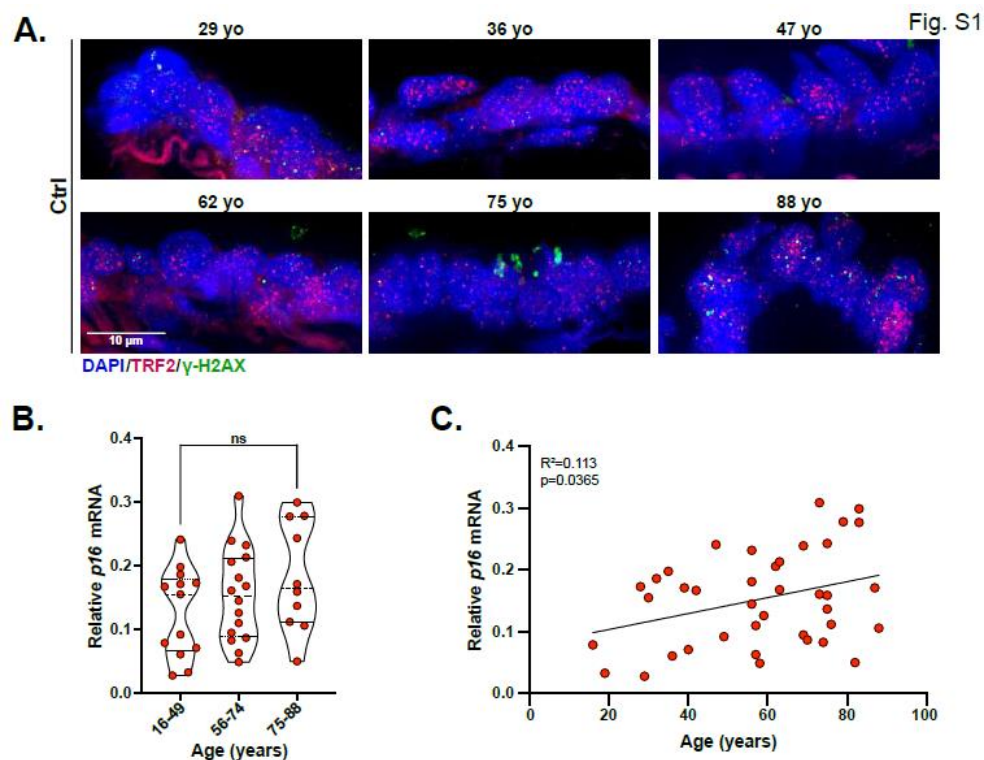
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Supplementary Data for Chapter II

Supplementary Figures

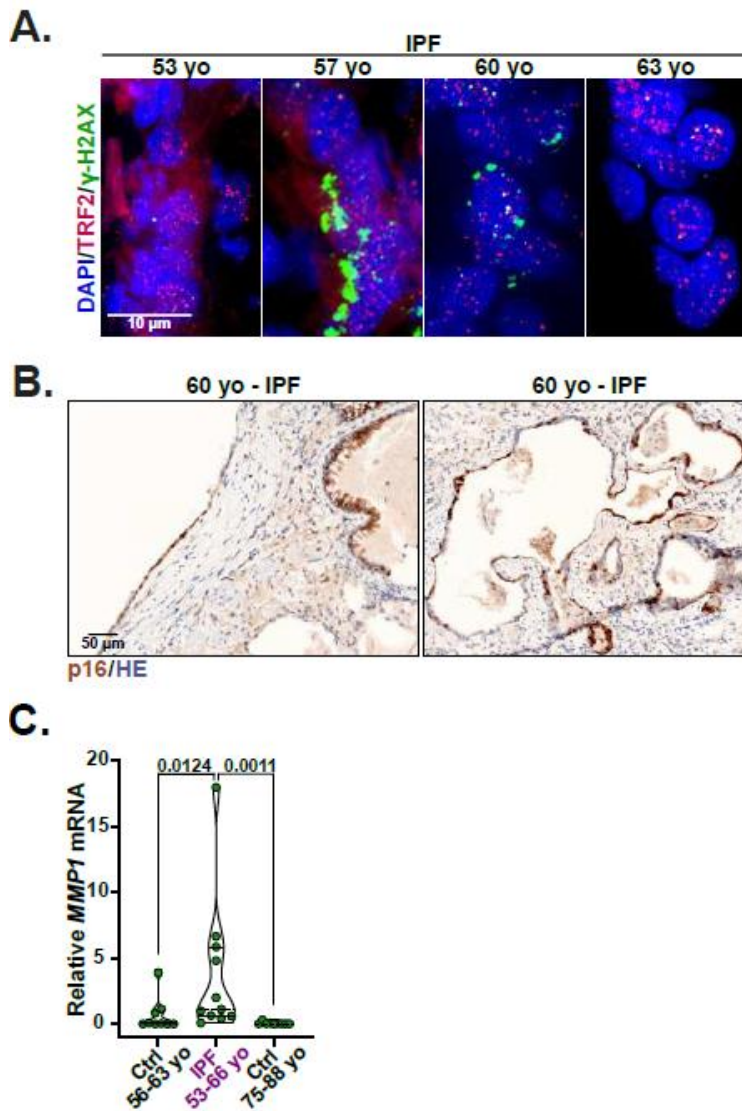
Figure S1.



Supplementary Figure 1. Senescence markers in aging human lungs. **A.** Representative images of TIF analysis in the lung epithelium from Ctrl donors at the indicated age. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Scale bar: 10 μ m. **B.** qRT-PCR analysis of p16 expression in lung samples of donors across the specified age ranges. p16 mRNA levels were normalized to ACTB mRNA and to one IPF lung sample used as control. Violin plots showing median, first and third quartile. Unpaired *t* test. ns: not significant. **C.** Linear regression analysis of p16 expression levels in lung samples relative to donor age.

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Figure S2.



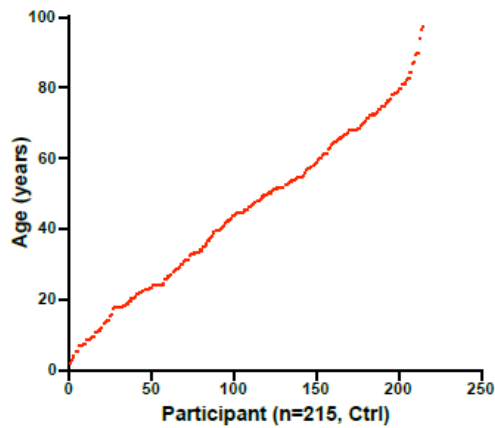
Supplementary Figure 2. Exacerbated cellular senescence in IPF lungs. A. Representative images of TIF analysis in the epithelium of IPF lungs from donors at the indicated age. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Scale bar: 10 μ m. **B.** Representative images of p16 staining by immunohistochemistry in IPF lung samples from two 60-year-old donors. HE: Hematoxylin-Eosin. Bar scale: 50 μ m. **C.** qRT-PCR analysis of *MMP1* expression in lung tissue samples from age-matched Ctrl and IPF donors, as well as Ctrl donors aged 75 and older. *MMP1*

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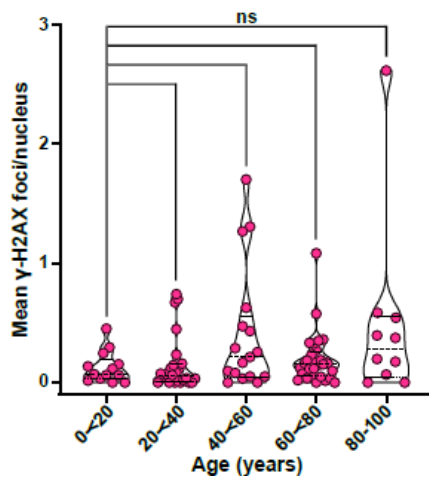
mRNA levels were normalized to *ACTB* mRNA and to one IPF lung sample used as control. Violin plots showing median, first and third quartile. Kruskal-Wallis test.

Figure S3.



Supplementary Figure 3. Age distribution of control donors providing nasal brushing samples.

Figure S4.



Supplementary Figure 4. No significant increase in total γ -H2AX signals in aging human nasal epithelial cells. Mean number of total γ -H2AX foci per nucleus in the nasal brushing samples of donors across the specified age ranges. On average, around 65 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal-Wallis test. ns: not significant.

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Supplementary tables

Table S1.

Sample	Age (years)	Sex	Comment
CTRL 1	16	M	Unused : artery rupture
CTRL 2	19	M	Declined : oedema
CTRL 3	28	F	Peripheral wedge biopsy prior to implantation
CTRL 4	29	M	Declined : contusion
CTRL 5	30	M	Declined : emphysema and adhesions
CTRL 6	32	F	Declined : mild emphysema
CTRL 7	35	M	Peripheral wedge biopsy prior to implantation
CTRL 8	36	M	Declined : infection
CTRL 9	39	M	Used for lobar transplant
CTRL 10	40	F	Used for lobar transplant
CTRL 11	42	M	Peripheral wedge biopsy prior to implantation
CTRL 12	47	F	Used for lobar transplant
CTRL 13	49	F	Peripheral wedge biopsy prior to implantation
CTRL 14	56	M	Declined : infection
CTRL 15	56	M	Used for single lung transplant
CTRL 16	56	M	Peripheral wedge biopsy prior to implantation
CTRL 17	57	F	Declined : embolus
CTRL 18	57	M	Declined : embolus
CTRL 19	58	M	Declined : infection
CTRL 20	59	M	Declined : emphysema
CTRL 21	62	F	Declined : infection
CTRL 22	63	M	Declined : contusion
CTRL 23	63	F	Peripheral wedge biopsy prior to implantation
CTRL 24	69	F	Declined : embolus
CTRL 25	69	F	Declined : contusion + emphysema
CTRL 26	70	F	Peripheral wedge biopsy prior to implantation
CTRL 27	73	M	Declined : emphysema
CTRL 28	73	M	Peripheral wedge biopsy prior to implantation
CTRL 29	74	M	Declined : contusion
CTRL 30	75	M	Declined : emphysema
CTRL 31	75	M	Declined : gastric tumor
CTRL 32	75	F	Peripheral wedge biopsy prior to implantation
CTRL 33	76	M	Peripheral wedge biopsy prior to implantation
CTRL 34	79	F	Declined : pleuritis on contralateral lung
CTRL 35	82	M	Declined : fragile lung
CTRL 36	83	M	Declined : secretions
CTRL 37	83	F	Peripheral wedge biopsy prior to implantation
CTRL 38	87	F	Peripheral wedge biopsy prior to implantation
CTRL 39	88	M	Peripheral wedge biopsy prior to implantation
IPF 1	53	M	Familial IPF

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IPF 2	55	M	Familial IPF
IPF 3	57	M	Sporadic IPF
IPF 4	60	M	Familial IPF
IPF 5	60	M	Sporadic IPF
IPF 6	61	M	Familial IPF
IPF 7	61	M	Familial IPF
IPF 8	61	M	Sporadic IPF
IPF 9	62	F	Familial IPF
IPF 10	62	M	Sporadic IPF
IPF 11	63	F	Sporadic IPF
IPF 12	66	F	Sporadic IPF

Supplementary Table 1. Lung samples used in this study.

Table S2.

	Age (years)	Sex	Nasal brushing	Olfactory test
CTRL	2,71	F	YES	NO
CTRL	5,27	M	YES	NO
CTRL	5,28	M	YES	NO
CTRL	6,96	M	YES	NO
CTRL	7,06	M	YES	NO
CTRL	7,06	M	YES	NO
CTRL	7,25	M	YES	NO
CTRL	7,43	M	YES	NO
CTRL	8,56	M	YES	NO
CTRL	8,69	M	YES	NO
CTRL	9,20	M	YES	NO
CTRL	9,40	M	YES	NO
CTRL	9,47	F	YES	NO
CTRL	10,66	F	YES	NO
CTRL	10,70	F	YES	NO
CTRL	11,36	M	YES	NO
CTRL	11,41	F	YES	NO
CTRL	12,01	F	YES	NO
CTRL	13,28	F	YES	NO
CTRL	13,85	F	YES	NO

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CTRL	14,01	M	YES	NO
CTRL	14,15	F	YES	NO
CTRL	15,51	F	YES	NO
CTRL	15,73	M	YES	NO
CTRL	17,53	F	YES	NO
CTRL	17,68	F	YES	NO
CTRL	17,79	M	YES	YES
CTRL	17,80	M	YES	YES
CTRL	17,94	M	YES	NO
CTRL	18,01	M	YES	NO
CTRL	18,15	F	YES	NO
CTRL	18,49	F	YES	NO
CTRL	18,78	F	YES	NO
CTRL	18,93	M	YES	NO
CTRL	19,54	M	YES	NO
CTRL	20,26	F	YES	NO
CTRL	20,56	F	YES	NO
CTRL	20,83	F	YES	NO
CTRL	21,57	M	YES	NO
CTRL	21,74	F	YES	NO
CTRL	21,87	F	NO	YES
CTRL	21,91	M	YES	NO
CTRL	22,22	F	NO	YES
CTRL	22,26	F	YES	NO
CTRL	22,28	F	NO	YES
CTRL	22,32	F	NO	YES
CTRL	22,61	M	YES	NO
CTRL	22,94	F	YES	NO
CTRL	22,94	F	YES	NO
CTRL	22,97	F	YES	NO
CTRL	23,42	F	YES	YES

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CTRL	23,47	F	YES	NO
CTRL	23,98	M	NO	YES
CTRL	24,04	M	YES	NO
CTRL	24,05	F	YES	NO
CTRL	24,08	M	YES	NO
CTRL	24,11	F	YES	NO
CTRL	24,12	F	YES	YES
CTRL	24,22	F	YES	NO
CTRL	24,35	F	YES	NO
CTRL	24,67	M	NO	YES
CTRL	25,12	F	NO	YES
CTRL	25,53	F	NO	YES
CTRL	25,79	F	YES	YES
CTRL	25,84	F	YES	NO
CTRL	26,08	M	NO	YES
CTRL	26,47	F	YES	NO
CTRL	26,57	M	YES	NO
CTRL	27,11	F	YES	YES
CTRL	27,80	F	YES	NO
CTRL	28,36	F	YES	NO
CTRL	28,64	F	YES	NO
CTRL	28,90	M	YES	NO
CTRL	28,91	F	NO	YES
CTRL	29,36	F	YES	YES
CTRL	29,89	F	YES	NO
CTRL	29,91	F	YES	NO
CTRL	30,03	F	NO	YES
CTRL	30,77	M	YES	NO
CTRL	31,07	F	NO	YES
CTRL	31,10	M	YES	NO
CTRL	31,21	F	YES	NO
CTRL	32,49	M	YES	NO
CTRL	32,91	F	YES	NO

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CTRL	32,98	M	YES	YES
CTRL	33,14	M	YES	NO
CTRL	33,32	M	YES	NO
CTRL	33,48	F	YES	NO
CTRL	33,49	F	YES	NO
CTRL	33,75	F	NO	YES
CTRL	33,99	M	YES	NO
CTRL	34,94	M	YES	NO
CTRL	35,05	F	YES	NO
CTRL	35,67	M	YES	NO
CTRL	36,61	F	YES	NO
CTRL	36,93	M	YES	NO
CTRL	37,69	M	YES	NO
CTRL	37,80	F	YES	YES
CTRL	39,00	F	YES	NO
CTRL	39,51	M	YES	NO
CTRL	39,78	F	YES	NO
CTRL	39,81	F	YES	NO
CTRL	40,13	F	YES	NO
CTRL	40,50	F	YES	NO
CTRL	40,93	F	YES	YES
CTRL	41,89	F	YES	NO
CTRL	42,32	F	YES	YES
CTRL	42,58	F	YES	NO
CTRL	43,08	F	YES	NO
CTRL	43,75	M	YES	NO
CTRL	43,99	F	YES	NO
CTRL	44,39	F	YES	NO
CTRL	44,56	M	YES	NO
CTRL	44,58	F	YES	NO
CTRL	44,62	F	YES	YES
CTRL	44,66	F	YES	YES

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CTRL	45,26	M	YES	NO
CTRL	45,32	F	YES	YES
CTRL	45,54	M	YES	NO
CTRL	46,43	M	YES	NO
CTRL	46,48	F	YES	NO
CTRL	46,86	M	YES	YES
CTRL	47,27	F	YES	NO
CTRL	47,61	F	YES	NO
CTRL	48,02	M	YES	NO
CTRL	48,20	F	YES	NO
CTRL	48,34	F	YES	NO
CTRL	49,03	M	YES	YES
CTRL	49,49	F	YES	NO
CTRL	49,79	F	YES	YES
CTRL	49,82	F	YES	YES
CTRL	50,23	M	YES	YES
CTRL	50,33	M	YES	YES
CTRL	50,79	M	YES	NO
CTRL	51,26	M	YES	YES
CTRL	51,33	M	YES	NO
CTRL	51,59	F	YES	NO
CTRL	51,65	F	YES	NO
CTRL	51,87	M	YES	YES
CTRL	51,88	M	YES	YES
CTRL	51,98	F	YES	NO
CTRL	52,46	M	YES	NO
CTRL	52,48	F	YES	NO
CTRL	52,91	M	YES	YES
CTRL	53,41	M	YES	YES
CTRL	53,64	F	YES	YES
CTRL	53,75	F	YES	NO
CTRL	53,80	M	YES	NO
CTRL	54,19	M	YES	NO

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CTRL	54,58	M	YES	NO
CTRL	54,58	F	YES	NO
CTRL	54,72	M	YES	YES
CTRL	55,26	F	YES	NO
CTRL	55,84	M	YES	NO
CTRL	56,87	F	YES	NO
CTRL	57,14	M	YES	NO
CTRL	57,34	F	YES	YES
CTRL	57,57	M	YES	NO
CTRL	58,16	F	YES	YES
CTRL	58,31	F	YES	NO
CTRL	58,74	F	YES	YES
CTRL	59,73	F	YES	NO
CTRL	59,98	M	YES	YES
CTRL	60,19	M	YES	YES
CTRL	60,80	F	YES	YES
CTRL	61,41	M	YES	NO
CTRL	61,45	F	YES	YES
CTRL	62,50	M	YES	YES
CTRL	63,24	M	YES	YES
CTRL	63,81	F	YES	YES
CTRL	64,20	M	YES	YES
CTRL	64,73	M	YES	YES
CTRL	64,89	M	YES	YES
CTRL	65,16	F	YES	YES
CTRL	65,69	F	YES	YES
CTRL	66,07	M	YES	YES
CTRL	66,24	M	YES	NO
CTRL	66,65	M	YES	YES
CTRL	66,65	M	YES	NO
CTRL	67,45	M	YES	NO
CTRL	67,94	M	YES	YES

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CTRL	67,95	F	YES	NO
CTRL	68,11	M	YES	NO
CTRL	68,16	F	YES	NO
CTRL	68,26	F	YES	NO
CTRL	68,44	F	YES	NO
CTRL	68,45	F	YES	YES
CTRL	69,40	F	YES	NO
CTRL	69,89	F	YES	NO
CTRL	70,33	F	YES	NO
CTRL	70,55	M	YES	NO
CTRL	71,53	F	YES	NO
CTRL	72,15	M	YES	NO
CTRL	72,21	F	YES	NO
CTRL	72,46	F	YES	NO
CTRL	72,48	F	YES	NO
CTRL	72,82	F	YES	NO
CTRL	73,09	M	YES	NO
CTRL	73,90	F	YES	NO
CTRL	74,06	F	YES	NO
CTRL	74,68	M	YES	NO
CTRL	74,74	M	YES	NO
CTRL	75,70	M	YES	NO
CTRL	76,27	M	YES	NO
CTRL	76,33	M	YES	NO
CTRL	76,95	M	YES	NO
CTRL	78,06	F	YES	NO
CTRL	78,06	M	YES	YES
CTRL	78,37	F	YES	YES
CTRL	78,84	F	YES	YES
CTRL	79,75	F	YES	NO
CTRL	79,92	F	YES	NO
CTRL	81,03	F	YES	YES

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

CTRL	81,10	F	YES	YES
CTRL	81,27	F	YES	YES
CTRL	82,41	M	YES	NO
CTRL	82,90	M	YES	NO
CTRL	84,49	M	YES	NO
CTRL	86,93	F	YES	YES
CTRL	87,43	M	YES	YES
CTRL	89,47	M	YES	YES
CTRL	89,88	F	YES	YES
CTRL	89,88	M	YES	NO
CTRL	94,01	M	YES	YES
CTRL	96,58	F	YES	YES
CTRL	97,35	F	YES	YES
TBD24- <i>TERT</i>	18,63	F	YES	YES
TBD23- <i>TERT</i>	22,03	F	YES	YES
TBD27- <i>TERT</i>	29,34	M	YES	YES
TBD33- <i>RTEL1</i>	40,2	F	YES	YES
TBD32- <i>TERT</i>	43,8	M	YES	YES
TBD25- <i>TERT</i>	48,54	F	YES	YES
TBD3- <i>TERT</i>	51,70	M	YES	YES
TBD21- <i>TERC</i>	56,2	M	YES	YES
TBD26- <i>RTEL1</i>	58,02	F	YES	YES
TBD1- <i>TERT</i>	60,81	F	YES	NO
TBD22- <i>RTEL1</i>	62,15	F	YES	YES

Supplementary Table 2. Volunteers for nasal brushing and/or olfactory tests.

Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

Table S3.

Target	Species	Reference
β -catenin	Rabbit	8480S, Cell Signaling Technology
CD68	Mouse	NB100-683B, Novus Biologicals
LAMP2	Mouse	66301-1-Ig, ProteinTech
Phospho-H2A.X (Ser139) (γ -H2AX)	Mouse	05-636, Millipore
TRF2	Rabbit	NB110-57130, Novus Biologicals
Mouse IgG (Alexa Fluor 647)	Donkey	A-31571, Invitrogen
Rabbit IgG (Alexa Fluor 488)	Goat	A-11008, Invitrogen

Supplementary Table 3. Antibodies used in this study. All antibodies were used in IF experiments.

Table S4.

Target	Forward primer	Reverse primer
ACTB	5'-GCTGGAAGGTGGACAGCGA	5'- TGTACGCCAACACAGTGCTG
CD68	5'-TCCAGGGAAGCTGTGAGGGT	5'-AGCCGAGAATGTCCACTGTGC
MMP-1	5'-CAGAGATGAAGTCCGGTTTTTC	5'-GGGGTATCCGTGTAGCACAT
p16	5'-GGAAGGTCCCTCAGACATCC	5'-TACGAAAGCGGGGTGGGTTG
p21	5'-GCAGACCAGCATGACAGATT	5'-GGATTAGGGCTTCCTCTTGGA

Supplementary Table 4. Primers used in this study. All primers were obtained from Eurogentec (Belgium).

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Telomere dysfunction and proteostasis decline define distinct pathways of cellular senescence in the human respiratory tract

GENERAL DISCUSSION AND PERSPECTIVES

DISCUSSION

In this project, we investigated Alzheimer's disease (AD) and biological aging through the olfactory neuroepithelium (ONE), a neuronal tissue that can be accessed using the nasal brushing technique. This approach was combined with the assessment of olfactory function using standardized tests, allowing the evaluation of different components of olfaction.

The aim of this work was to address two complementary questions. First, to determine to what extent olfactory dysfunction and alterations of the olfactory system reflect the propagation of tau pathology during the course of AD. Second, to characterize the biological mechanisms involved in age-related cellular senescence within the ONE.

By combining histopathological, biological, and functional approaches, this project follows a translational perspective aimed at improving our understanding of both AD pathophysiology and the biological processes of aging. The study of the ONE and olfactory function thus provides a means to explore the relationship between cellular alterations and functional manifestations, using an accessible human model in living subjects.

I. Olfactory system alterations in Alzheimer's disease as a tool to understand tau pathology spreading

Olfaction is one of the earliest sensory functions affected during AD [173, 174]. Numerous studies have shown that a progressive decline in olfactory performance can be observed before the onset of cognitive impairment [174]. Although often not spontaneously reported by patients, this dysfunction represents a major early symptom and reflects functional changes within brain regions involved in olfactory processing.

Accordingly, the olfactory cortex is now recognized as one of the earliest brain regions to present accumulation of hyperphosphorylated tau protein, before the pathology progressively spreads to adjacent cortical regions during the stages described by Braak [116-118]. In addition to these cortical structures, other components of the olfactory system, such as the ONE, have also been investigated in the context of AD and have shown the presence of tau-related pathological signatures [183-185]. The literature indicates that the olfactory bulb

(OB) is affected at early stages of the disease, whereas the ONE has been less extensively studied and mainly at late stages [179, 182].

At present, it remains unclear whether tau pathology originates in the OB and subsequently spreads retrogradely to peripheral olfactory structures, or instead begins in the ONE and propagates anterogradely to the OB. This question is relevant as the "olfactory vector hypothesis" proposes that the nose may serve as an entry point for environmental or infectious agents, triggering tau aggregation in the ONE before its spreading to the OB, followed by adjacent cortical regions [209]. Supporting a possible spreading mode of tau pathology through the anterograde pathway, a recent study in a transgenic mouse model of tau pathology reported that, if experimentally induced in the ONE, tau accumulation may be able to spread to the OB [186]. Whether this applies to the naturally-occurring tau pathology in humans is however still highly debated and a recent study demonstrated tau propagation from medial temporal lobe structures toward the peripheral olfactory system [179], thus challenging the theory that human tau pathology spreads due to infectious or environmental agents from the ONE.

1. Histopathological characterization of tau pathology within the olfactory system

Our analyses of *post-mortem* tissues from the OB and the ONE at different disease stages indicated that tau pathology follows a spatio-temporal gradient consistent with Braak staging, extending later toward peripheral olfactory structures. At early stages (I-III), neurofibrillary tangles (NFTs) are predominantly detected in the inner layers of the OB and progressively spread to the more superficial layers at advanced stages (V-VI), where olfactory sensory neuron axon terminals reside (**Figure 14**). In parallel, no tau tangles were observed within the cell bodies of olfactory sensory neurons, even at advanced stages of the disease. These findings support the hypothesis of a tau pathology propagating from central cortical regions toward peripheral olfactory structures, likely through retrograde spreading along the olfactory pathway.

Moreover, our analysis of nasal brushing samples did not support the hypothesis that the ONE serves as a primary initiation site for tau pathology but instead favored the notion of a peripheral extension of a neurodegenerative process originating in the central nervous system. Based on these findings however, and contrary to our initial expectations, nasal brushing does not appear to be a relevant tool for the early diagnosis of AD.

2. Olfaction as an early and sensitive marker of tau pathology

Consistent with the histopathological analyses, our olfactory testing results support a spatio-temporal propagation of tau pathology within the olfactory system. Odor identification appears to be the earliest affected function, with detectable deficits already present at the preclinical stage. This impairment is more pronounced in preclinical individuals with abnormal tau measure, suggesting early involvement of central olfactory regions. Olfactory discrimination declines at a prodromal stage, when cognitive symptoms begin to emerge. This function, which depends on the integrity of the OB and associated cortical circuits, becomes altered as tau pathology progressively affects the different layers of the bulb. In contrast, the olfactory detection threshold was significantly reduced only at the demented stage compared with age-matched controls. This late impairment is consistent with peripheral involvement of the ONE and correlates with elevated levels of phosphorylated tau in nasal brushing samples from demented patients (**Figure 14**).

Our data support the potential of targeted olfactory assessments as complementary biomarkers for early AD. Among the sixteen odors tested in our cohort, the orange odor was particularly difficult to identify at the preclinical stage. As the disease progresses, additional odors also become harder to identify. This evolution suggests that the progressive alteration of specific brain connections, particularly within cortical regions involved in odor identification, may underline this functional decline. These observations support the use of olfactory testing as a tool for early detection. While a limited odor panel is not sufficient on its own to define a robust pathological profile, expanding and refining olfactory test batteries could allow the identification of

specific odor profiles that help support or exclude the presence of an underlying pathology.

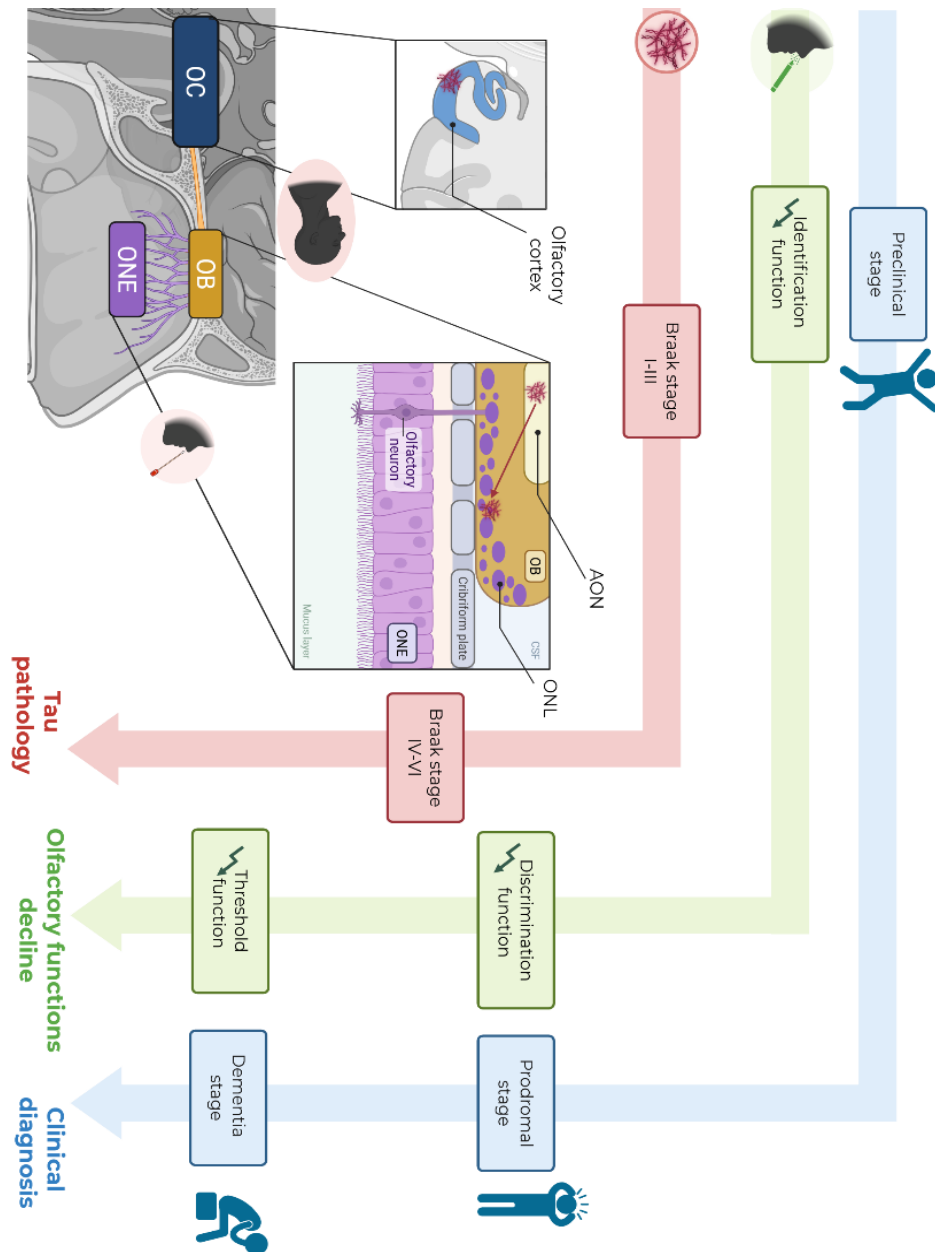


Figure 14. Spatio-temporal progression of tau pathology and olfactory dysfunction across Alzheimer's Disease stages. Schematic representation of the main findings of the project. Histopathological analyses indicate a progressive involvement of the olfactory system

by tau pathology, extending from central olfactory regions toward more peripheral structures across increasing Braak stages. In parallel, clinical manifestations are associated with a progressive decline in olfactory function. Early alterations affect central olfactory functions, such as odor identification, followed by impairments in discrimination, and finally by increased detection thresholds, reflecting later involvement of peripheral olfactory structures. Together, these observations support the concept that olfactory dysfunction mirrors the anatomical progression of tau pathology within the olfactory system. AON: anterior olfactory nucleus; OB: olfactory bulb; OC : olfactory cortex; ONE: olfactory neuroepithelium; ONL: olfactory nerve layer; CSF: cerebrospinal fluid. Generated by biorender.

II. Olfaction tools to study aging mechanisms

The detection threshold is not only altered in patients with AD at the demented stage but is also the first olfactory function to decline during normal aging, before discrimination and identification. Therefore, the combined analysis of functional and biological data from the ONE is particularly relevant for studying aging mechanisms, which are addressed in the second part of this work.

1. Aging-related changes in the olfactory neuroepithelium

Our analyses relied on the same combination of approaches to explore another aspect of human biology, namely cellular aging. The study of aging mechanisms is complex due to the heterogeneity of the markers involved, whose expression strongly depends on the tissue considered, its cellular composition, and its vulnerability to age-related changes [210, 211].

The analysis of cells obtained from nasal brushing samples collected from participants of different ages, as well as from patients with telomeropathies, allowed the identification of cellular senescence markers directly within the human ONE. The results show that this tissue exhibits a progressive increase in senescence markers with age, particularly those associated with impaired proteostasis. This alteration is reflected by an increase in SA- β -gal activity and an increased lysosomal content (**Figure 15**).

These findings indicate that, despite its continuous regenerative capacity, the ONE is sensitive to the effects of biological aging. They suggest that senescence mechanisms gradually develop within this tissue, affecting its ability to maintain cellular homeostasis.

2. Olfaction as a mirror of biological aging

With aging, the ONE undergoes progressive changes in its structure and cellular function [212, 213]. These changes are reflected not only at the biological level but also at the functional level [212, 213]. Although this tissue retains regenerative activity throughout life, these alterations indicate a gradual decline in its ability to maintain optimal function.

The decline of olfactory function is a well-described feature of physiological aging [213]. It mainly manifests as a progressive reduction in olfactory sensitivity, whereas discrimination and identification functions are generally affected at later stages. This functional evolution is consistent with the biological changes observed in the ONE during aging.

The combined analysis of olfactory performance and senescence markers helped identify which biological mechanisms are most closely associated with age-related sensory decline. A significant correlation was observed between the olfactory detection threshold and β -gal activity, an enzymatic marker of cellular senescence. This association suggests that olfactory neuroepithelial cells with increased lysosomal activity contribute directly to the reduction in olfactory sensitivity observed in older individuals (**Figure 15**).

In contrast, no correlation was found between olfactory performance and damaged telomeres. This absence of association indicates that senescence mechanisms related to telomere instability are not a major determinant of olfactory functional decline in this tissue.

Taken together, data obtained in the two distinct parts of this PhD thesis indicate that olfaction reflects both neurodegenerative processes and mechanisms of physiological aging.

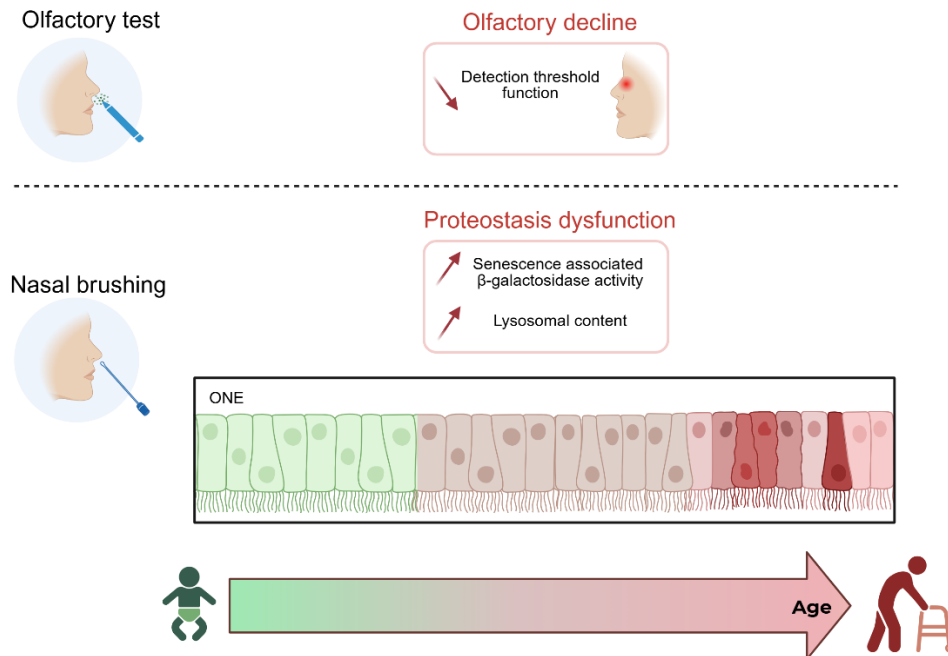


Figure 15. Age-related cellular senescence in the olfactory neuroepithelium is associated with reduced olfactory sensitivity. Schematic representation of age-related biological and functional changes in the olfactory neuroepithelium (ONE). During normal aging, the olfactory detection threshold is the earliest function to decline. This functional alteration is associated with progressive cellular senescence within the ONE, characterized by increased senescence-associated β -galactosidase activity and elevated lysosomal content, reflecting impaired proteostasis. These findings indicate that the ONE reflects biological aging processes at both cellular and functional levels. Generated by biorender.

III. Values of olfactory-based approaches in fundamental research and disease monitoring

In a research context, nasal brushing and exfoliation of the ONE offer several major advantages. This approach is minimally invasive and does not require anesthesia or surgical procedures, in contrast to nasal biopsies or cerebrospinal fluid sampling. It can be performed in living subjects and repeated over time, making it compatible with longitudinal studies. Importantly, they provide access to cellular material directly involved in aging and neurodegenerative processes. Compared with *post-mortem* studies, this approach allows the investigation of active biological processes rather than frozen states,

and offers an alternative to animal models, which do not fully reproduce the cellular and molecular complexity of the human brain. Access to the ONE in living humans therefore represents an opportunity to study the biological mechanisms involved in aging and neurodegenerative diseases (NDDs).

Cells obtained by nasal brushing are suitable for molecular and genetic analyses and can, to a certain extent, be cultured *in vitro* to investigate their functional properties. This is particularly relevant in the context of neurodegeneration (ND), as it allows the direct study of neurons derived from living patients and the investigation of pathological mechanisms at the cellular level.

Nasal brushing also represents a valuable tool for genetic analysis. Collected cells can be used for sequencing approaches to identify mutations present in the olfactory tissue. This is especially useful when blood cells are no longer informative, such as in patients with telomeropathies who have undergone bone marrow transplantation. Nasal brushing therefore provides access to genetic information without the need for invasive or complex procedures.

Overall, nasal brushing appears to be a relevant approach for exploring tissue-specific biological mechanisms of aging and NDDs in living humans. It allows the combination of cellular, molecular, and functional analyses within an accessible model that is directly relevant for human research but also in a clinical context.

Indeed, due to their minimally invasive nature, nasal brushing and olfactory testing show strong potential for clinical implementation. They provide complementary information and can be performed rapidly, making them compatible with the practical limits of patient care.

Beyond their interest for clinical evaluation, olfactory-based approaches are also relevant for disease monitoring. Longitudinal follow-up of biological and functional parameters would allow the evaluation of disease progression over time, the identification of transitions toward more advanced stages, and a more refined classification of pathological stages. This approach would also make it possible to monitor patients' responses to treatment by integrating both molecular changes and functional outcomes.

In the long term, these approaches could be integrated as complementary tools alongside those already used in clinical practice. They would provide both functional and biological assessments that are minimally invasive and easy to implement, thereby contributing to improved management of NDDs.

IV. Limitations of the thesis project

Based on all participants and samples analyzed, this work provided informative results regarding AD as well as the mechanisms of biological aging. However, the main limitation of this study lies in the relatively small size of the cohorts included in both projects. This constraint limits the statistical power of the analyses and requires cautious interpretation of the results. It also highlights the need to validate these findings in larger cohorts. In addition, some conclusions remain partial, as interindividual variability in olfactory performance and biological markers has not yet been fully integrated into the analyses. This heterogeneity will need to be taken into account in future studies in order to refine the interpretation of the observed relationships. Finally, part of the analyses relied on cross-sectional data, which limits the assessment of temporal changes in the studied parameters. This limitation is largely explained by time constraints of the project, as a substantial portion of the work was devoted to optimizing experimental approaches and establishing scientific protocols. Longitudinal studies will therefore be necessary to complement and strengthen the conclusions presented here.

Additional methodological limitations must be considered when interpreting the data. One of the main limitations of using nasal brushing lies in the cellular heterogeneity of the collected samples. Indeed, the sampling procedure involves passing through the respiratory epithelium, leading to the collection of multiple cell types. As a result, the specific analysis of olfactory neurons partly depends on their variable proportion within the samples, which may introduce a bias related to the number of neurons available for analysis.

Ultimately, it is important to emphasize the limited availability of ONE biopsies, particularly from patients spanning the full AD continuum. Observations based on nasal brushing do not fully recapitulate the

ONE, as they capture only cells directly accessible to the brush and not those located in the lamina propria, where tau accumulation have previously been reported in AD patients.

V. Future research opportunities

Several methodological perspectives can be considered, including more advanced transcriptomic and proteomic approaches to screen for possible early biomarkers of AD in nasal brushing samples. Such large-scale analyses are also of interest in the context of aging. Integrating transcriptomic and proteomic analyses would possibly make it possible to identify new markers and to explore more precisely the mechanisms of physiological and pathological aging. Notably, elucidating the biological basis of the marked variability in proteostasis dysfunction observed across our cohort of aged individuals would be of great interest, as it could lead to the identification of novel cellular mechanisms underlying aging.

The implementation of longitudinal studies, following participants over several years, would allow a better characterization of the temporal dynamics of the studied markers and their evolution in relation to clinical progression. By observing the same individuals over time, it would become possible to distinguish interindividual variability from changes truly related to aging or disease onset, thereby improving the interpretation of the clinical and research potential of these tools. In addition, comparing results obtained from nasal brushing with blood-based biomarkers would make it possible to assess the concordance, sensitivity, and specificity of these approaches. This methodological complementarity could provide a more comprehensive view of patients' biological status, particularly in older individuals or those at risk, and strengthen the predictive value of these tools in clinical follow-up.

In this project, the analyses primarily focused on tau pathology. Extending the analysis to other PTMs of tau, such as acetylation or ubiquitination, could be of particular interest. As tau phosphorylation alone remains insufficiently specific to distinguish AD from other NDDs, combining multiple tau-related molecular signatures in nasal

DISCUSSION

brushing samples may improve the specificity of early detection and differential diagnosis during the preclinical stages of AD. Moreover, extending these investigations to amyloid pathology would be highly relevant. *Post-mortem* studies have shown the presence of amyloid deposits in the olfactory neuroepithelium of patients with AD. Additionally, amyloid pathology is closely associated with neuroinflammatory processes, which may occur very early in the pathological cascade and therefore deserve further investigation using this model.

Finally, integrating these methods into therapeutic trials targeting early disease stages would allow a more sensitive detection of biological effects of interventions before the appearance of clear clinical symptoms. These tools could thus serve as monitoring biomarkers, facilitating the evaluation of treatment efficacy and the early adjustment of therapeutic strategies.

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APPENDIX

Olfactory decline tracks central-to-peripheral spread of tau pathology in Alzheimer's disease

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Abstract

INTRODUCTION: Olfactory decline often precedes cognitive symptoms in Alzheimer's disease (AD) and is linked to tau pathology. Yet, whether tau aggregates start in peripheral olfactory structures or spread from central regions remains debated.

METHODS: We analyzed 34 *post mortem* human olfactory bulb (OB) and 2 neuroepithelium (ONE) samples across Braak stages, combined with ex vivo nasal swabs and standardized olfactory testing in 88 clinically characterized participants.

RESULTS: Tau aggregates appeared in the anterior olfactory nucleus layer of the OB from Braak stage III, spreading to peripheral layers only in late stages. Olfactory identification declined in the preclinical phase and was linked to central tau pathology. Discrimination worsened during the prodromal stage, while threshold impairment appeared only in dementia, reflecting the anatomical progression of tau pathology.

DISCUSSION: This anatomical-functional link supports a central-to-peripheral spread of tau pathology in the olfactory system, with stage-specific deficits suggesting targeted smell tests as early AD biomarkers.

KEYWORDS

Alzheimer's disease, nasal brushing, olfaction, olfactory bulb, olfactory neuroepithelium, tau pathology

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Highlights

- Tau aggregates appear in the internal anterior olfactory nucleus layer of the human olfactory bulb (OB) at Braak stage III.
- The olfactory nerve layer of the OB and the olfactory neuroepithelium become involved only at advanced Braak stages.
- Olfactory subdomain impairments mirror the central-to-peripheral progression of tau pathology.
- Specific odor identification is impaired as early as the preclinical stage.

1 | BACKGROUND

Alzheimer's disease (AD) is a mixed proteinopathy characterized by extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau.^{1,2} Both pathologies contribute to neurodegeneration and cognitive impairment,¹ but NFT burden shows a closer correlation with cognitive decline^{1,3,4} and more strongly predicts symptom onset.^{2,5–7} NFTs emerge through pathological tau transformation, in which excessive phosphorylation induces conformational changes that promote misfolding, oligomer formation, and subsequent aggregation.⁸ These oligomers progressively assemble into paired helical filaments,¹ which first appear as neuropil threads within axons and later evolve into pre-tangles in the perinuclear cell body.⁹ With disease progression, these structures mature into NFTs that disrupt neuronal integrity, impair synaptic communication, and compromise network function.³ Even after neuronal death, ghost tangles persist,⁹ highlighting their stability and underlining the role of tau aggregation as a progressive and self-perpetuating driver of AD pathology.

Tau pathology, widely regarded as the most reliable marker of AD progression, is believed to originate early in life in the locus coeruleus, later spreading to the transentorhinal cortex during Braak stage I.¹⁰ By Braak stage II, the olfactory bulb (OB), entorhinal cortex (EC), and hippocampus begin to exhibit pathological changes.¹⁰ Notably, during Braak stages I and II, cognitive functions remain intact. However, olfactory dysfunction often arises in AD before the onset of overt cognitive impairment and mostly impacting odor identification ability.^{11–16} This olfactory decline has been linked to pathological tau aggregates in the EC at early stages, and in the OB, more specifically in the anterior olfactory nucleus (AON) layer, around Braak stages II to III.^{17–20}

Despite the established correlation between olfactory dysfunction and AD progression, it is still unclear whether the olfactory system plays a primary role in the development of the disease. The "olfactory vector hypothesis" proposes that the olfactory system may serve as an entry point for environmental or infectious agents that trigger cortical neurodegeneration.^{21–23} Supporting this model, preventing the accumulation of pathological forms of tau in the olfactory neuroepithelium (ONE) through its recurrent stripping in the mouse model expressing the human P301S mutant form of tau, markedly reduces pre-tangle-like tau pathology in the piriform cortex.²⁴

In humans, evidence for early tau pathology in the ONE is limited and inconsistent. A few studies have reported tangle-like profiles in the ONE tissue of AD cases^{25–27} yet these observations were made predominantly in patients at advanced stages of the disease. Conversely, some studies reported little to no tau labeling in pathological conditions, with occasional staining also observed in non-AD or control individuals.^{28–31} Contrasting conclusions drawn by these studies contribute to the uncertainty surrounding whether the human ONE exhibits tau pathology, particularly at the earliest stages of AD. Moreover, a recent study convincingly demonstrated tau propagation from medial temporal lobe structures toward the peripheral olfactory system,³² challenging the theory that tau pathology occurs in the ONE due to infectious or environmental agents and eventually spreads to connected region. These opposing hypotheses nevertheless underscore the complexity and potential bidirectionality of tau spreading within the olfactory pathway, as well as possible differences between humans and mice.

Determining whether tau pathology originates in the olfactory cortex or peripheral olfactory structures is crucial for developing early interventions for AD. The primary objective of this study was to test the directionality of tau pathology propagation within the human olfactory system. To address this objective, we combined histopathological analyses of *post mortem* olfactory tissues with *ex vivo* nasal epithelial samples from individuals spanning the AD clinical continuum, together with olfactory assessments. This integrated approach enabled us to map the anatomical progression of tau pathology, supporting a trajectory from the central nervous system to peripheral olfactory structures. It also underscored the value of combining biological and functional measures to capture early disease dynamics.

2 | METHODS**2.1 | Post mortem human samples**

Human *post mortem* OB tissues were obtained from the Netherlands Brain Bank (NBB), with anonymized clinical summaries of the donor's medical record and histopathological brain analysis (Table S1 in supporting information). Two *post mortem* human OB tissues from body donors at Braak stage II were obtained from UCLouvain body dona-

tion program (ethical approval: UCL-2020-355; Table S1). In total, OB samples from 34 patients were used ($F = 20$, $M = 14$; 81.0 ± 15.1 years). All presented tauopathy ranging from Braak stage I to Braak stage VI (I: $n = 3$, II: $n = 5$, III: $n = 5$, IV: $n = 5$, V: $n = 4$, VI: $n = 12$). Braak staging was determined by the brain banks according to a widely accepted neuropathological standard³³ based on phosphorylated tau (p-tau) immunohistochemistry (IHC) using the AT8 antibody (P-tau Ser202/Thr205). Assessments were performed by a single rater on sections from the hippocampus and EC, temporal associative cortex, and occipital cortex. Braak stages were interpreted as a continuum reflecting the progressive spread of NFTs from transentorhinal regions to the occipital cortex, with hippocampal involvement generally observed from stage II onward. For 15 patients, both frozen and fixed tissues were available; for 17 patients, only frozen tissues were available; and for 2 patients, only fixed tissues were obtained (Table S1). Frozen bulb samples were mechanically homogenized and split into two fractions for different analyses. Two *post mortem* human ONE from body donors at Braak stage II were also obtained from UCLouvain body donation program ($F = 1$, $M = 1$; 92.5 ± 2.5 years, ethical approval: UCL-2020-355).

2.2 | Participants recruitment for ex vivo study

Participants were recruited through clinical neurology consultations and from clinical studies led by Prof. Bernard Hanseuw and Prof. Caroline Huart at Cliniques Universitaires Saint-Luc, the academic hospital of UCLouvain. This study was performed according to the Declaration of Helsinki and approved by the medical ethics committee of the academic hospital of UCLouvain (ethical approvals: UCL-2016-121 and UCL-2010-148). The 88 participants ($F = 45$, $M = 43$) provided written informed consent (Table S2 in supporting information). An oral questionnaire was run to exclude individuals meeting predefined exclusion criteria: known cause of olfactory disorder, acute or chronic rhinosinusitis, history of head trauma, participation in a clinical trial involving an AD treatment, or a medical history of major psychiatric conditions or other neurological disorders. The participants' diagnoses described below relied on an examination of amyloid and tau status with either tau positron emission tomography (PET) imaging with [^{18}F]-MK6240 plus 3D T1 magnetic resonance imaging (MRI; $n = 37$),³⁴ amyloid PET ($n = 57$), cerebrospinal fluid (CSF) analyses ($n = 19$), or blood plasma analyses ($n = 9$). Clinical diagnosis were based on the Mini-Mental State Examination (MMSE; $n = 88$) and a neuropsychological examination ($n = 57$), which included testing of four cognitive domains (verbal episodic memory, executive function, language, and visuospatial function), described in the supplementary Methods and Table S3 in supporting information.

2.3 | Evaluation of amyloid and tau status

Amyloid positivity was determined by either CSF analysis, blood analysis, or by amyloid PET with [^{18}F] flutemetamol or [^{11}C] Pittsburgh

RESEARCH IN CONTEXT

1. **Systematic review:** The prevailing model of tau pathology propagation in Alzheimer's disease (AD), based on Braak staging, posits a "prion-like" mechanism characterized by progressive transsynaptic spread from central brain regions to more peripheral cortical areas. Because olfactory deficits frequently precede cognitive symptoms, some have proposed that tau pathology may originate in peripheral regions, such as the olfactory neuroepithelium, and spread centrally toward the olfactory bulb and cortex. This theory is supported by the fact that the olfactory neuroepithelium is directly exposed to environmental stressors, including toxins and pathogens. However, this view has been challenged by studies demonstrating a trajectory consistent with central-to-peripheral spread.
2. **Interpretation:** In this study, we combined cellular, molecular, and functional approaches to determine whether the olfactory system is a primary target or a secondary site of involvement in the progression of AD. Our analysis encompassed *post mortem* human olfactory tissues and ex vivo nasal epithelial cells from individuals across different clinical stages, together with corresponding assessments of olfactory function. Our results support a retrograde model of tau pathology propagation, from the olfactory cortex to the olfactory neuroepithelium via the olfactory bulb. These results challenge the notion that the human olfactory epithelium serves as the initial site of tau pathology onset.
3. **Future directions:** This work supports the considerable potential of olfactory testing in AD, both for improving diagnosis and for refining disease staging and monitoring.

compound B as described previously.^{35,36} PET values were expressed on the Centiloid scale; blood plasma amyloid beta ($\text{A}\beta_{42}/\text{A}\beta_{40}$ and CSF $\text{A}\beta_{42}$ levels were measured with automated assays.^{35–37} Participants were classified as amyloid positive (A+) if Centiloid > 26, plasma soluble $\text{A}\beta_{42}/\text{A}\beta_{40}$ ratio ≤ 0.0465 , or CSF $\text{A}\beta_{42} < 437$ pg/ml.^{36,38–40} Tau pathology was assessed with [^{18}F]MK-6240 PET (Lantheus Inc.), a second-generation tracer for tau tangles as described previously.⁴¹ Radiosynthesis was performed at KULeuven and doses of 185 ± 5 MBq were administered intravenously. Ninety minutes post-injection, a 30-minute dynamic acquisition was performed on a Philips Vereos digital PET CT. Images were reconstructed with the manufacturer's algorithm (attenuation, scatter, decay correction, time of flight, point spread function modeling, and 1 mm reslicing). Tau PET scans were co-registered with a 3D T1 MRI for visual analysis and the Braak-like stage was reported for each participant. Participants were classified as tau positive (T+) if the visual Braak-like stage was > 0.

TABLE 1 Individuals enrolled in the study for nasal brushings and olfactory tests.

Category	A- CN	A+ CN	Prodromal AD	AD dementia
Number of participants	29	15	28	13
Mean age (years)	70.4	73.6	70.8	75.2
Sex (F:M)	(15:14)	(8:7)	(13:15)	(8:5)
Amyloid testing (A)	A-	A+	A+	A+
Tau testing (T)	T-	T- or T+	T+	T+
Global cognitive Z score (SD)	≥ -1.5	≥ -1.5	< -1.5	< -1.5
MMSE (/30)	> 24	> 24	> 24	≤ 24

Notes: Summary of demographic and diagnostic data for individuals enrolled in the study, including clinically normal participants and patients at preclinical, prodromal, or advanced stages of dementia. Diagnoses were based on comprehensive clinical evaluation, neuropsychological testing, brain imaging (positron emission tomography), blood samples and cerebrospinal fluid analysis when available.

Abbreviations: AD, Alzheimer's disease; CN, cognitively normal; MMSE, Mini-Mental State Examination; SD, standard deviation.

2.4 | Clinical classification of participants

Participants were classified as either cognitively normal (CN; $n = 47$, all cognitive domain z scores ≥ -1.5 and MMSE > 24) or cognitively impaired (CI; $n = 41$, at least one cognitive domain z score < -1.5). Among CN participants, 44 had available amyloid testing and were further subdivided into two subgroups with either normal ($n = 29$, A-) or abnormal ($n = 15$ A+) amyloid status, while 3 CN participants had no available amyloid or tau biomarker data. The A+ CN group comprises preclinical AD patients with either normal ($n = 8$ T-) or abnormal ($n = 7$ T+) tau PET. To focus specifically on AD, only A+ CI individuals were included. A+ CI participants were further subdivided in two subgroups based on disease severity with either prodromal AD (PR AD; $n = 28$) or AD dementia ($n = 13$; Table 1 and S2).

2.5 | Olfactory tests

Olfactory tests were performed using the Sniffin' Sticks test (Burghart) validated by Hummel et al.⁴² This test includes three subtests yielding three scores: threshold (T), discrimination (D), and identification (I). Odors were delivered using pen-like devices with tips infused with an odorant solution. The tip was held 2 cm below the nose and moved slowly from the left to right nostril for > 3 to 4 seconds. The threshold test consisted of randomized triplets of pens numbered 1 to 16. In each triplet, only one pen contained the odorant (phenylethanol), with pen no. 1 containing the most concentrated solution. The odorant was serially diluted (1:2) down to pen no. 16. For each triplet, the subject was asked to identify the odor-containing pen in a three-alternative forced-choice (3-AFC) paradigm with eyes closed. The test starts with the lowest concentration, and a staircase paradigm is used, by which two consecutive correct answers led to a lower concentration in the next triplet and one incorrect answer led to a higher concentration in the next triplet. This procedure is repeated until seven turning points are identified. The T score was the mean of the last four turning points in the staircase, ranging from 1 to 16. The discrimination test presented 16 triplets in random order: two pens contained the same odor, while

the third contained a different odor. Subjects identified the different odor in a 3-AFC paradigm, eyes closed. The D score was the number of correct responses (0–16). Sixteen pens were presented individually; for each, subjects were asked to identify the odor based on a four-alternative forced choice procedure from four illustrated options read aloud by the examiner. The I score was the number of correct identifications (0–16). The final TDI score was the sum of the three individual scores (range 1–48).

2.6 | Nasal brushing and cell isolation

Nasal brushing was performed by a trained ear, nose, and throat specialist using two flocked swabs (Copan, FLOQSwabs 503CS01) introduced into both olfactory clefts at the level of the middle turbinate under endoscopic guidance. The ONE was exfoliated from both nostrils by rotation. One swab was placed in 1 mL of 4% paraformaldehyde; the other in 1 mL chilled Dulbecco's Modified Eagle Medium/F12 (Thermo Fisher Scientific, Gibco 11039021) with 10% fetal bovine serum (Cytiva, SV30160.03), 1% penicillin/streptomycin solution (Capricorn Scientific, PS-B), protease inhibitors (1:25; Roche, 11836170001), and phosphatase inhibitors (1:20; Roche, 4906837001). After vortexing for 15 seconds, the swab was removed from the fixative. The cell-containing solution was centrifuged 15 minutes at $300 \times g$, fixative discarded, pellet resuspended in phosphate-buffered saline (PBS), and cytocentrifuged (Cytospin 4, Thermo Fisher Scientific) for 10 minutes at $200 \times g$ onto slides (Immuno-Cell, 61.100.59). The swab in culture medium was rubbed against another swab to detach cells. Samples were then divided into two aliquots before centrifugation at 4°C for 45 seconds at $20,630 \times g$. Supernatants were removed and cell pellets were frozen at -80°C .

2.7 | IHC

Ten-micrometer sections of paraffin-embedded post mortem OB tissues were prepared using a microtome (Microm Microtech, HM 340E).

The sections were deparaffinized by incubating them in two successive xylene baths (5 minutes each), followed by two 2-minute immersions in 100% isopropanol. Endogenous peroxidases were inactivated in 0.3% H₂O₂/methanol for 30 minutes. After rinsing with distilled water and delineation of tissue areas using a DakoPen (Sigma-Aldrich, Z377821), sections were washed three times for 10 minutes in Tris-buffered saline (TBS), then blocked for 1 hour at room temperature (RT) in 10% normal goat serum (NGS; Sigma-Aldrich, S26-M) and incubated overnight with primary antibodies (Table S4 in supporting information). Secondary antibody incubation was carried out for 30 minutes at RT. Negative controls omitted primary antibodies. Sections were washed three times for 10 minutes in TBS, incubated with streptavidin-horseradish peroxidase (Vector Laboratories, SK-4103) for 30 minutes, rinsed three times 10 minutes, and developed with 3,3'-diaminobenzidine (Vector Laboratories, PK-7100): 2 minutes for PHF1/AT8, 1 minute 30 seconds for AT270/olfactory marker protein (OMP). Reactions were stopped in distilled water. Sections were dehydrated through graded isopropanol baths (70%–100%, 4 × 2 minutes) and finally mounted with distyrene, plasticizer, and xylene (Sigma-Aldrich, 1.00579.0500). Visualization was conducted using a ZEISS Axioskop 40 microscope; images were captured with ZEISS ZEN Blue 2.5 and analyzed in ImageJ2.⁴³

2.8 | Immunofluorescence

For IF experiments, *post mortem* OB paraffin blocks were sectioned at 5 µm. Sections were deparaffinized and rehydrated through a series of xylene, isopropanol, ethanol, and distilled water washes, then rinsed in 1 × PBS. Antigen retrieval was performed by heating in boiling citrate buffer (10 mM, 0.1% Tween, pH 6) for two 5-minute cycles, followed by a 20-minute cooldown at RT. Permeabilization was performed in Tris-HCl buffer with 0.5% Triton X-100 for 1 hour at 37°C. After 1 × PBS rinses, non-specific binding was blocked for 45 minutes at RT using 1 × PBS supplemented with 0.1% Triton X-100, 0.21% bovine serum albumin (BSA) and 2.1% NGS. For *ex vivo* nasal brushing cells, antigen retrieval was performed with two successive incubations in boiling citrate buffer (1 minute followed by 3 minutes), followed by 30 minutes of permeabilization at RT. Blocking was then carried out for 1 hour at RT in 1 × PBS containing 0.1% Triton X-100, 0.21% BSA, and 2.1% normal horse serum (NHS). Samples were incubated overnight at 4°C with primary antibodies (Table S4). Negative controls omitted primary antibodies. The next day, samples were washed and incubated for 40 minutes at RT with secondary antibodies (Table S4). After final washes with 1 × PBS, slides were mounted in fluorescence mounting medium (Agilent, S3023) containing 0.1 µg/mL 4',6-diamidino-2-phenylindole (Sigma-Aldrich, D9542). Visualization was done using a ZEISS Axio Observer Z1 confocal microscope; images were acquired with ZEISS ZEN Blue 2.6 and analyzed in ImageJ2.⁴³ Low-magnification (2X) images were obtained with a Panoramic Confocal scanner (3DHISTECH).

2.9 | Enzyme-linked immunosorbent assay

Post mortem OB and *ex vivo* smear samples were lysed in a buffer containing 150 mM NaCl, 20 mM Tris, 1% NP-40 (Sigma, 74385-1L), and 10% glycerol, supplemented with protease inhibitors (1:25, Roche, 11836170001) and phosphatase inhibitors (1:20, Roche, 4906837001). Mechanical lysis was performed using a syringe (Terumo Agani, 15438652; BD Plastipak, 303172). After centrifugation for 10 minutes at 20,000 × g at 4°C, the supernatant was collected and protein concentration was determined using the Bradford assay. P-tau at Thr181 (p-tau181) and total tau were quantified using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, KHO0631; KHB0041) per manufacturer's instructions. All assays were run in duplicate. Protein input was maximized, averaging 20 µg/well. Detection limits were 15.6 pg/mL for p-tau181 and 31 pg/mL for total tau. P-tau181 levels were normalized to total tau for each sample.

2.10 | Statistical analyses

Figures and statistical analyses were performed using GraphPad Prism 10. Normality was evaluated using the Shapiro–Wilk test ($\alpha = 0.05$). For two-group analyses, a Student *t* test was used when normality was met; otherwise, the Wilcoxon test was applied. For multiple group comparisons, parametric one-way analysis of variance was used for normally distributed data, otherwise the Kruskal–Wallis test was applied.

3 | RESULTS

3.1 | NFT-like aggregates accumulate in the *post mortem* OB from Braak stage III onward

In the OB and AON, a relay structure that connects the bulb to the olfactory cortex, NFTs and neuropil threads have been previously reported to emerge from Braak stage II to III onward.^{17,18} Accordingly, using IHC, we observed small amounts of NFT-like aggregates in all 13 *post mortem* OB samples from donors with Braak stage III or higher, with their abundance progressively increasing at more advanced stages (Figure 1A and Figure S1 in supporting information). The flame-shaped aggregates characteristic of NFTs were, however, not observed in OB samples from patients at Braak stage I or II (Figure 1A and Figure S1). Similar observations were made with the PHF1 antibody (P-tau Ser396/Ser404), a later-stage marker more specific to the mature forms of pathological aggregates and specifically highlighting flame-shaped NFT-like aggregates (Figure 1A).

Consistent with previous studies,^{17,44–48} we observed that, from Braak stage III onward, NFTs accumulate in the AON layer of the OB, which we identified using an anti-calbindin antibody, a marker of interneurons primarily located in the AON layer (Figure 1B). In contrast, the outer layer of the OB, known as the olfactory nerve layer

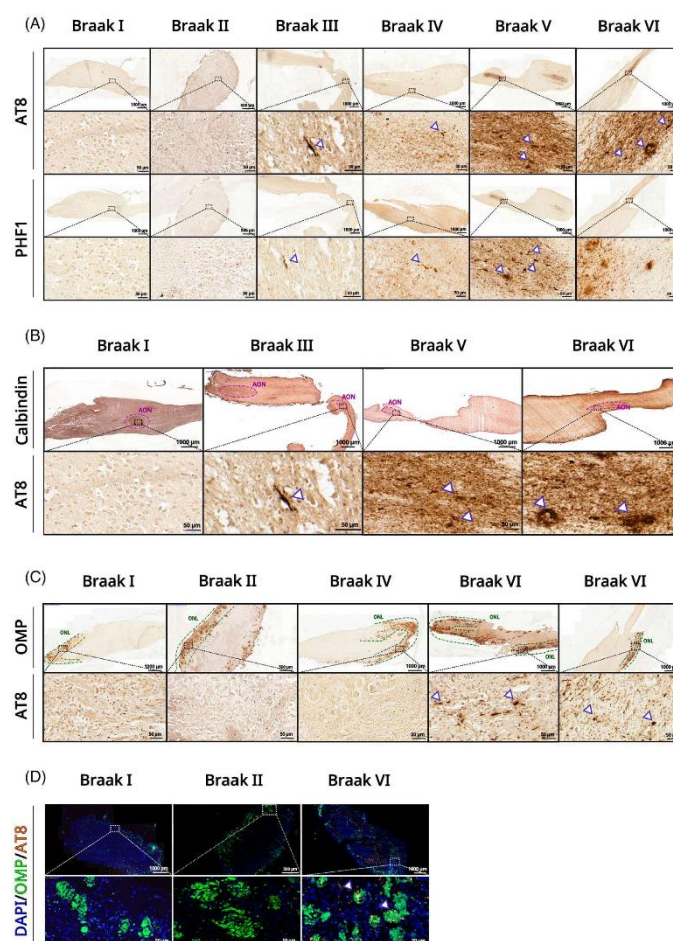


FIGURE 1 Distribution of NFT-like aggregates in human olfactory bulbs across Braak stages. A, Representative pictures of paraffin sections immunostained for AT8 (Ser202/Thr205) or PHF1 (Ser396/Ser404) in OB samples from patients at the indicated Braak stages ($n = 6$). Above: Low magnification view, scale bar: 500 or 1000 μm , as indicated. Below: Higher magnification of the highlighted region, scale bar: 50 μm . Flame-shaped NFTs (arrowheads) were not observed at Braak stages I or II. B, Above: The AON region in the OB samples ($n = 4$) was identified using calbindin immunolabeling (purple dotted lines), scale bar: 1000 μm . Below: Higher magnification of the highlighted region, scale bar: 50 μm . Arrowheads indicate NFT-like aggregates. Co-detection of the ONL and NFTs in OB samples ($n = 5$) from donors at indicated Braak stage using OMP and AT8 antibodies by IHC (C) or IF (D) ($n = 3$; lower panel with OMP in green, AT8 in red, and DNA in blue). Above: Low magnification view, scale bar: 500 or 1000 μm , as indicated. Below: Higher magnification of the highlighted region, scale bar: 50 μm . Co-localization events are indicated by arrowheads. AON, anterior olfactory nucleus; IF, immunofluorescence; IHC, immunohistochemistry; NFT, neurofibrillary tangle; OB, olfactory bulb; OMP, olfactory marker protein; ONL, olfactory nerve layer.

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(ONL), identified using an antibody against the OMP, remained unaffected at Braak stage IV (Figure 1C). However, AT8-positive NFT-like aggregates appeared by stage VI, indicating a delayed onset of NFTs in the ONL compared to the AON layer in AD patients (Figure 1D).

Together, our findings support the idea that although NFTs emerge early in the AON layer, their accumulation in the ONL happens during the advanced stages of the disease.

3.2 | P-tau181-positive deposits accumulate in the AON from Braak stage I onward

Having found that NFTs appear in the OB of AD patients from Braak stage III, we next assessed the spatiotemporal progression of pathological tau filaments known as neurofibrillary threads, specifically labeled by the AT270 antibody targeting p-tau181, a modification widely used as a quantifiable biomarker in human CSF and known to appear earlier in the progression of tau pathology than p-tau205.⁴⁹ We detected tau deposits possibly corresponding to neurofibrillary threads in the AON layer as early as Braak stage I, with a progressive increase in their accumulation at advanced stages (Figure 2A). Notably, the early distribution of these aggregates appeared to be layer specific. Specifically, in Braak I samples in which the earliest neurofibrillary threads were observed in the inner layers of the olfactory bulb, we also noted the absence of these aggregates in the OMP-positive layers corresponding to the ONL (Figure 2B). These findings support the notion that tau aggregation, from the formation of neurofibrillary threads to the emergence of the first NFTs, initially develops in inner layers such as the AON before extending to more peripheral layers such as the ONL. Consistent with the AT8 and PHF1 stainings however, NFT-like aggregates only became detectable with this antibody at Braak stage V and were found clustering in the AON layer (Figure 2A). The results obtained by immunostaining were confirmed by ELISA as we found a significant increase in the p-tau181/total tau ratios at advanced Braak stages (V/VI) compared to early stages (I/II; Figure 2C).

Together, this spatiotemporal progression of tau immunoreactivity across Braak stages supports a model in which the pathology progresses from the AON layer to the rest of the OB.

3.3 | NFT-like lesions are not detected in exfoliated cells from the ONE in living humans

To further investigate the progression of tau pathology within the olfactory system, we next examined the presence of NFTs in the ONE. Previous studies on AD pathology in the ONE have primarily examined *post mortem* tissues from individuals with late-stage dementia, limiting the ability to assess the presence of tau pathology in the ONE at earlier Braak stages.^{27,28} Due to the limited availability of *post mortem* ONE tissues, we adopted an *ex vivo* approach, analyzing exfoliated cells collected from nasal brushing of living individuals. These samples were collected from a cohort representing the full clinical spectrum of AD, including amyloid-negative CN individuals (A- CN, $n = 29$), as well as patients at preclinical stage, CN, but with amyloid pathology (A+ CN,

$n = 15$). This cohort also included CI patients at the prodromal (PR AD, $n = 28$), or dementia (AD dementia, $n = 13$) stages of AD (Table 1 and S2).

First, to determine whether nasal smears contain mature olfactory sensory neurons, we performed immunofluorescence against OMP, using *post mortem* ONE tissue as a positive control for the staining (Figure 3A). Next, we tested a total of 26 nasal smear samples ($n = 9$ CN, $n = 9$ PR AD, and $n = 8$ AD dementia) and observed OMP staining in $\approx 11\%$ of the collected cells (Figure 3A), with a pattern closely resembling previously reported observations.⁵⁰ After validating the nasal brushing approach for studying the ONE, we proceeded to assess tau pathology. First, we confirmed the presence of total tau in the dendritic cilia of nasal smear cells, mirroring the distribution observed in the ONE tissue (Figure 3A). However, AT8 immunoreactivity was absent in OMP-positive neurons collected via nasal smear, even in individuals with dementia, suggesting that NFTs do not form in these cells (Figure 3B).

These findings indicate that NFT-like lesions do not form in olfactory sensory neurons, supporting the hypothesis that ONE is unlikely to serve as the entry point for tau pathology in humans.

3.4 | Increased levels of p-tau181 in nasal smears from patients with AD dementia

Given the apparent lack of NFT-like lesions in nasal brushing, even in dementia patients, we next examined the samples for potential differential enrichment of p-tau181. We first attempted to measure p-tau181 levels in nasal smear extracts using ELISA. While total tau was readily detectable, p-tau181 levels fell below the assay's detection threshold (Figure S2A-B in supporting information). We therefore turned to immunostaining, using *post mortem* ONE tissue from a Braak stage II case as a positive control (Figure 4A). To assess p-tau181 levels specifically in OMP⁺ cells within the nasal smears, we performed co-staining with AT270 (p-tau181) and OMP antibodies (Figure 4A). For each sample, the ratio of OMP⁺/p-tau181⁺ cells was compared to the ratio of OMP⁺/total tau⁺ cells (Figure 3A and 4A). Analysis of samples from CN ($n = 9$), PR AD ($n = 9$), and AD dementia patients ($n = 8$) revealed a pattern consistent with that observed in the OB: a significant increase in tau phosphorylation in AD dementia cases ($p = 0.037$), and a trend toward increased p-tau181 in the prodromal group ($p = 0.23$; Figure 4B).

Together, these data support the results obtained with the OB samples and the hypothesis that human tau pathology does not start in the ONE.

3.5 | Olfactory identification declines in the preclinical stage, while detection threshold drops only in AD dementia

Early impairment in odor identification has been previously linked to tau aggregates in primary olfactory regions such as the piriform cortex, AON, amygdala, and EC.³² It remains unclear whether threshold

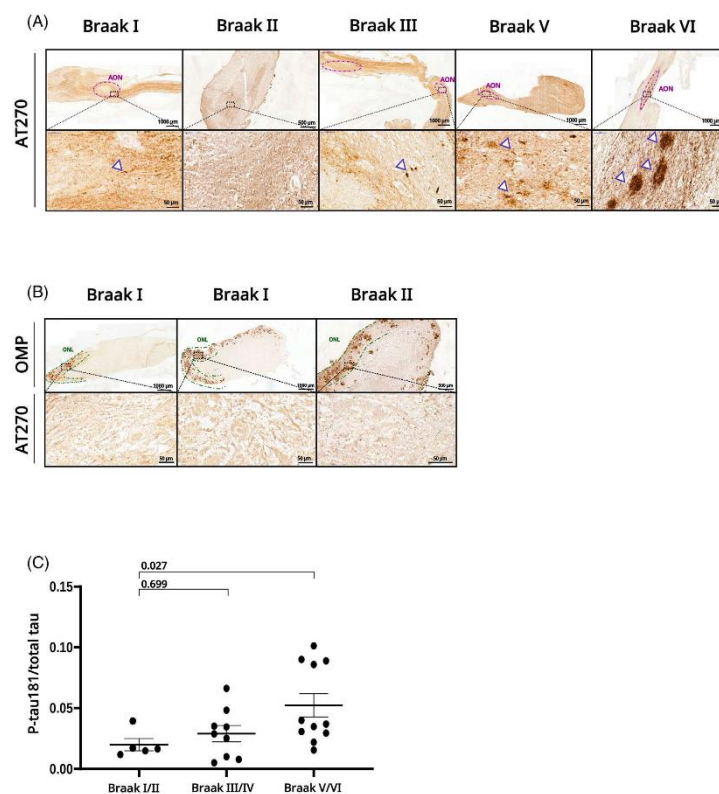


FIGURE 2 Detection of p-tau181 in human olfactory bulbs across Braak stages. A, Representative pictures of paraffin sections immunostained with the AT270 antibody (p-tau181). Above: Low magnification view, scale bar: 500 or 1000 μ m, as indicated. The position of the AON is indicated with purple dotted lines. Below: Higher magnification of the highlighted region, scale bar: 50 μ m. Tau deposits appear as early as Braak stage I while NFT-like aggregates become detectable only from Braak stage V onward (arrowheads) in the OB samples ($n = 5$). B, Co-detection of the ONL and neuropil threads aggregates in OB samples ($n = 3$) from donors at indicated Braak stage using OMP and AT270 antibodies by IHC. Above: Low magnification view, scale bar: 500 or 1000 μ m, as indicated. Below: Higher magnification of the highlighted region, scale bar: 50 μ m. C, Quantification of p-tau181 and total tau levels by enzyme-linked immunosorbent assay in OB tissue extracts from patients at the indicated Braak stages ($n = 25$). Mean $\pm$ standard error of the mean, Mann-Whitney statistical test. AON, anterior olfactory nucleus; IHC, immunohistochemistry; NFT, neurofibrillary tangle; OB, olfactory bulb; OMP, olfactory marker protein; ONL, olfactory nerve layer; p-tau, phosphorylated tau.

performance—generally considered an indicator of peripheral olfactory function—is impacted in the early stages of AD pathology.⁵¹ To investigate this further and to gain additional insight into the direction of tau pathology spread, we evaluated olfactory function using the standardized Sniffin' Sticks test in an age-matched cohort encompassing various clinical stages of AD (Table 1 and S2). This test evaluates

three distinct components of olfactory ability: identification (I), but also threshold (T) and discrimination (D), each with a maximum score of 16.

We found that the overall TDI score declined significantly at the prodromal ($p = 0.041$) and dementia ($p < 0.0001$) stage of AD compared to A–CN volunteers (Figure 5A). When analyzing the three subcomponents individually, the T score showed a significant decline

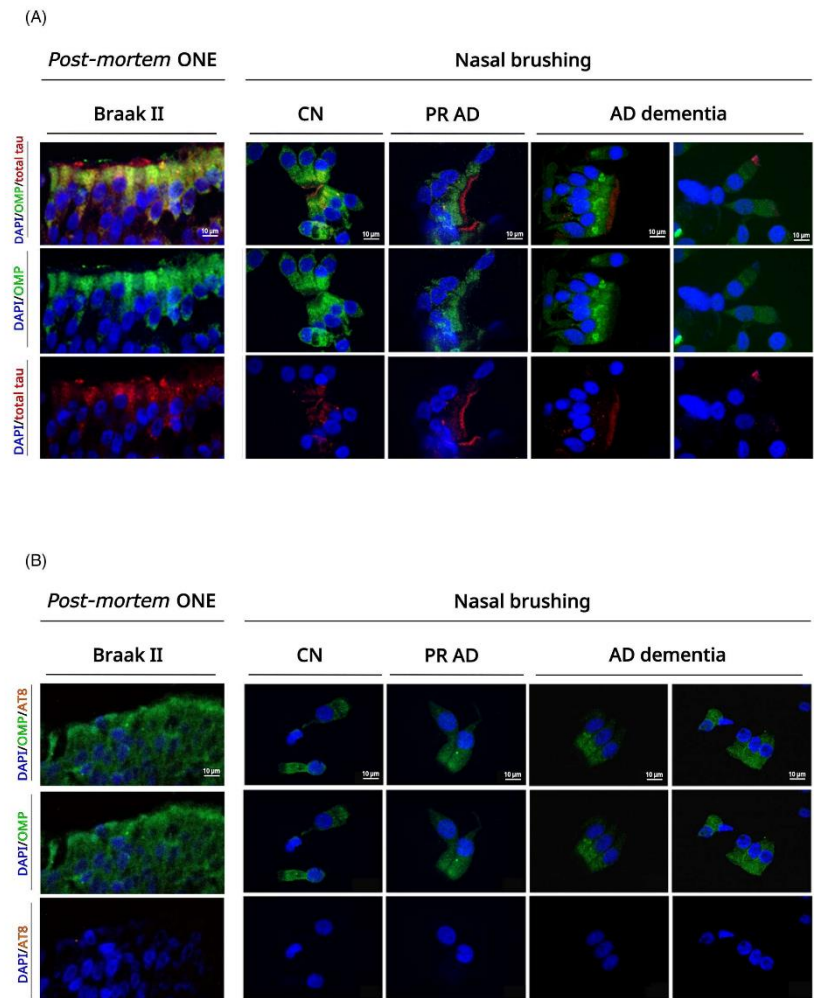


FIGURE 3 Detection of NFT-like aggregates in human olfactory neurons from olfactory neuroepithelium and ex vivo nasal smears. A, Representative pictures of immunofluorescence staining using OMP (green) and tau (red) antibodies. DNA, stained with DAPI (blue), marks nuclei. Left: *post mortem* ONE sample from a patient at Braak stage II ($n = 1$); right: representative pictures of nasal smear cells ($n = 4$) from CN donors or AD patients at the prodromal (PR AD) or the dementia stage of AD (AD dementia). Scale bar: 10 μm . B, Same as (A) using OMP (green) and AT8 (red) antibodies. Scale bar: 10 μm . AD, Alzheimer's disease; CN, cognitively normal; DAPI, 4',6-diamidino-2-phenylindole; OMP, olfactory marker protein; ONE, olfactory neuroepithelium.

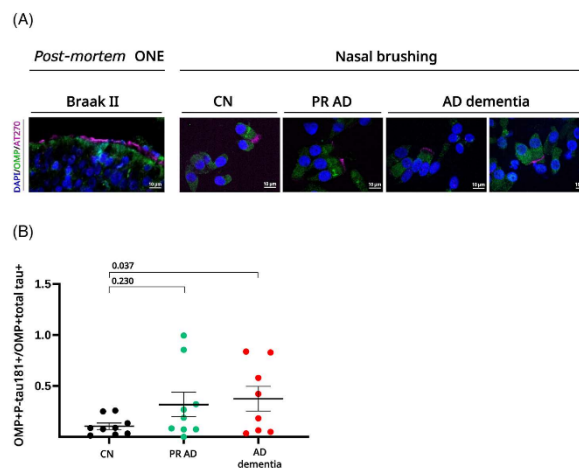


FIGURE 4 Detection of p-tau181 in human olfactory neurons from olfactory neuroepithelium and ex vivo nasal smears. A, Representative pictures of immunofluorescence staining using OMP (green) and AT270 (P-tau181) (red) antibodies. DNA, stained with DAPI (blue), marks nuclei. Left: post mortem ONE sample from a patient at Braak stage II ($n = 1$); right: representative pictures of nasal brushing cells ($n = 4$) from CN donors or AD patients at the prodromal (PR AD) or the dementia stage of AD (AD dementia). Scale bar: 10 μ m. B, Quantification of the ratio of OMP⁺/p-tau181⁺ to OMP⁺/tau⁺ cells across CN participants ($n = 9$), prodromal AD patients ($n = 9$), and AD dementia patients ($n = 8$). Mean $\pm$ standard error of the mean, unpaired t, and Mann-Whitney statistical tests. AD, Alzheimer's disease; CN, cognitively normal; DAPI, 4',6-diamidino-2-phenylindole; OMP, olfactory marker protein; ONE, olfactory neuroepithelium; p-tau, phosphorylated tau.

only at the dementia stage ($p < 0.0001$; Figure 5B). In contrast, D ability was already significantly impaired at the prodromal stage ($p = 0.0125$; Figure 5C). Consistent with previous findings,^{15,16,52,53} the I score followed a different trajectory, with a significant reduction evident as early as the preclinical stage in A+ CN individuals ($p = 0.004$), and continued to decline through the prodromal and dementia stages (Figure 5D). A+ CN patients can be either negative or positive for tau pathology (Table 1). To further explore whether the early deficit in identification performance may be linked to tau pathology, we stratified A+ CN patients into two subgroups: those with a positive amyloid PET, but negative tau PET (A+T-; $n = 8$), and those with both amyloid and tau PET positivity (A+T+; $n = 7$). The results revealed a significant decline in identification scores exclusively in A+T+ CN patients compared to A- CN participants ($p < 0.0001$), supporting an early association between olfactory dysfunction and tau accumulation in primary olfactory regions (Figure 5E).

Consistent with the p-tau181 staining results, the decline in threshold performances was only seen at the dementia stage of AD, with no reduction at earlier stages (Figure 5B). This prompted us to test whether the late decline in threshold performances may be related to the increased OMP⁺/p-tau181⁺ to OMP⁺/tau⁺ ratio that we observed in nasal smear cells in late disease stages (Figure 4A-B). Consistently, we observed a trend toward an inverse correlation ($R^2 = 0.39$;

$p = 0.003$) between threshold scores and the ratio of OMP⁺/p-tau181⁺ to OMP⁺/tau⁺ cells in nasal smears (Figure 5F), whereas the correlation with discrimination scores was notably weaker ($R^2 = 0.15$; $p = 0.087$; Figure S3 in supporting information).

Together, these data support the hypothesis that early olfactory impairment in AD, marked by defective identification ability, stems from tau pathology in the central nervous system, which later spreads to the olfactory nasal epithelium and impacts detection threshold.

3.6 | Identification of orange odor is significantly impaired from the preclinical stage

Earlier work using the UPSIT (University of Pennsylvania Smell Identification Test) assay of odor identification has associated pathological tau accumulation in the brain with impaired identification of certain specific odors.³² Notably, the scent of orange was strongly associated with early tau deposition in olfactory regions during Braak stages II and III, while the scent of leather was linked to tau accumulation at Braak stages III to IV.³²

This prompted us to analyze participants' responses to each of the 16 odors in the Sniffin' Sticks identification test (Figure 6A). To focus

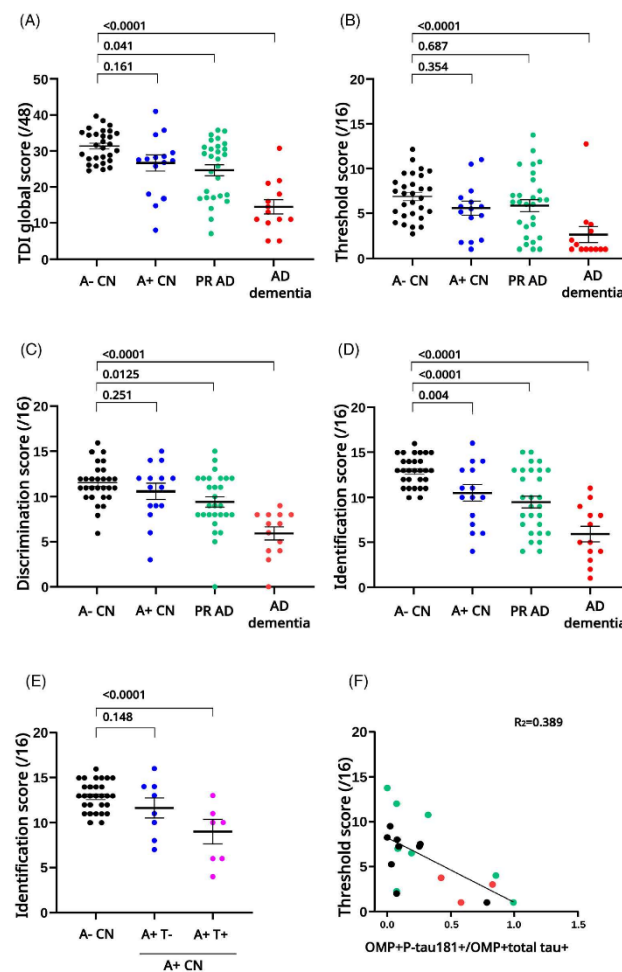


FIGURE 5 Olfactory dysfunction correlates with tau pathology across AD stages. A, The composite TDI score was determined using the standardized Sniffin' Sticks test in $n = 29$ A- CN ($F = 15$, $M = 14$; mean age = 70.4 years) and $n = 15$ A+ CN ($F = 8$, $M = 7$; mean age = 73.6 years) participants, $n = 28$ prodromal AD (PR AD; $F = 13$, $M = 15$; mean age = 70.8 years) patients, and $n = 13$ patients at the dementia stage of AD (AD dementia; $F = 8$, $M = 5$; mean age = 75.2 years). B, Threshold scores in the same cohort. C, Discrimination scores in the same cohort. D, Identification scores in the same cohort. E, Identification scores in $n = 29$ A- CN and $n = 15$ A+ CN participants subclassified into $n = 8$ A+ T- (purple) and $n = 7$ A+ T+ (pink). A-E, Mean $\pm$ standard error of the mean, Kruskal-Wallis and ordinary one-way analysis of variance statistical tests. F, Linear regression analysis between olfactory threshold scores and the ratio of OMP+ p-tau181+ to OMP+ tau+ cells established in Figure 4B on nasal smears. A, amyloid; AD, Alzheimer's disease; CN, cognitively normal; OMP, olfactory marker protein; p-tau, phosphorylated tau; T, tau; TDI, threshold/discrimination/identification.

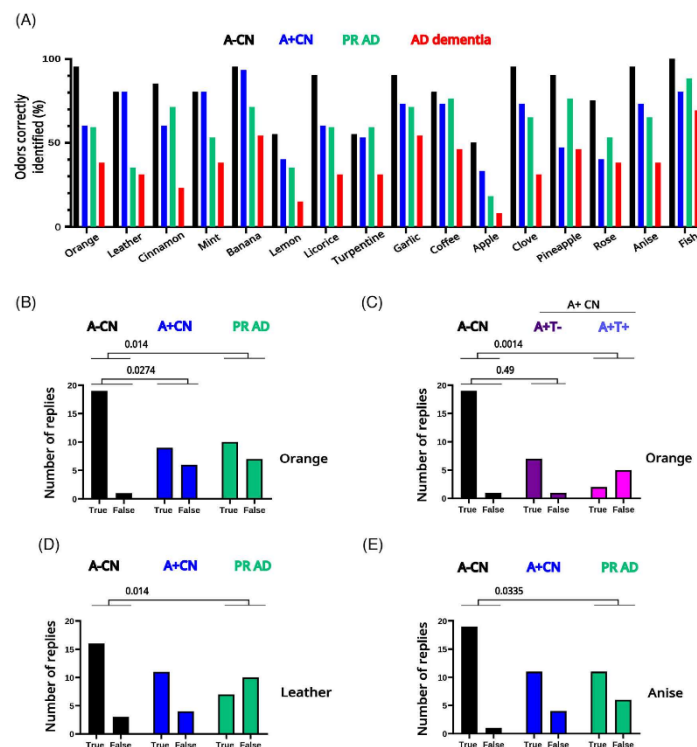


FIGURE 6 Differential impairment of odor identification across AD stages. A, Percentage of odors correctly identified by the participants for the 16 odors in the Sniffin' Sticks test was calculated as a percentage of total true responses in $n = 20$ A-CN (black) and $n = 15$ A+CN participants (blue), $n = 17$ prodromal AD (PR AD) patients (green), and $n = 13$ patients at the dementia stage of AD (dementia AD; red). B, Number of replies for the orange scent in $n = 20$ A-CN (black), $n = 15$ A+CN (blue), and $n = 17$ PR AD (green). C, Number of replies for the orange scent in $n = 20$ A-CN (black), $n = 8$ A+T- CN (purple), and $n = 6$ A+T+ CN (pink) participants. True: correct answer; False: wrong answer. D, E, Same as (B) for the indicated scents. B-E, Fisher exact tests. A, amyloid; AD, Alzheimer's disease; CN, cognitively normal; T, tau.

on the most informative odors within the A+CN group, we applied predefined selection criteria: (1) odors were required to have a correct identification rate of at least 80% in the A-CN group, thereby excluding odors with low baseline performance in CN individuals; (2) identification performance in the A+CN group had to show a reduction of at least 30% compared to the A-CN group; and (3) this reduction had to be consistent across the AD continuum. Based on these criteria, identification of the orange scent was significantly impaired from the A+CN preclinical stage ($p = 0.0274$; Figure 6B). We further compared by stratifying the A+CN group and observed that the significant alteration in orange identification was actually driven by

A+T+ patients ($p = 0.0014$; Figure 6C). Following the same criteria, identification of leather and anise scents showed a delayed decline, with impairments appearing only from the prodromal stage onward (Figure 6D-E).

Collectively, the presence of selective odor identification deficits in the preclinical phase without accompanying threshold impairments suggests that the olfactory system is more likely affected downstream in AD pathogenesis, rather than serving as an initial trigger. Notably, the early deficit in orange odor identification, limited to T+ individuals within the CN group, further supports the involvement of tau pathology in this downstream effect.

4 | DISCUSSION

4.1 | Overview

The reference model of tau propagation in AD, based on Braak staging, describes a "prion-like," trans-synaptic spread from central to peripheral cortical regions.^{12,54} As olfactory deficits often precede cognitive symptoms, some authors have proposed an ascending prion-like propagation model initiated in peripheral structures such as the ONE and progressing toward the OB and cortex.^{16,55,56} This model is supported by the vulnerability of the ONE, which is directly exposed to environmental agents and connected to the OB without blood-brain barrier protection.^{22,52,57} However, this hypothesis has been challenged by studies reporting a central-to-peripheral trajectory, with tau emerging in medial temporal and entorhinal regions before involving olfactory cortices and peripheral structures.^{32,58,59}

Thus, the actual dynamics of tau pathology in the olfactory system remain controversial, notably because most available data derive from *post mortem* tissues at advanced stages, making it difficult to infer the true chronology of spread.^{26,27} In this study, we combined cellular, molecular, and functional approaches to explore whether the olfactory system plays a primary or secondary role in the etiology of AD.

4.2 | Propagation of tau pathology in the olfactory system

We demonstrate that in AD, NFT accumulation initially appears in the AON layer and extends to the ONL only at more advanced Braak stages (V–VI). In parallel, no tangles were detected in neuronal cell bodies of the ONE, either in nasal smears or in two *post mortem* ONE samples (Braak stage II). Nevertheless, nasal smears from patients at the dementia stage showed elevated p-tau181 levels. These findings may appear to contrast with previous reports describing tau pathology in the ONE, including tau-positive neuronal cell bodies.^{26,27} However, those observations are largely based on *post mortem* samples obtained at advanced stages of AD, and to date no study has demonstrated the presence of tau aggregates in the ONE at very early stages of the disease. Moreover, when tau pathology is reported in the ONE, aggregates are predominantly detected in supporting structures such as the lamina propria rather than within the neuroepithelial layer itself.³⁰ This suggests that nasal brushing, which does not collect such structures, may not be optimally suited for detecting pathological tau in the ONE. Future analyses of intact ONE tissues will be required to better characterize tau pathology across Braak stages.

Our findings on OB samples nevertheless align with previous reports indicating early NFT presence in the OB.^{17–20,60,61} The sequential involvement of OB layers supports a temporal spread pattern. Given that the olfactory cortex is affected at Braak stage I,¹⁰ the most consistent interpretation is a central-to-peripheral propagation toward the ONE via retrograde transport.⁶² However, it remains to be determined whether this progression reflects a prion-like mode of propagation or instead arises from differential cellular vulnerability across OB layers. Given that studies in transgenic mouse models such

as PS19 reported a propagation of tau from the periphery toward the central nervous system along the olfactory anterograde pathway,²⁴ it is also possible that, under specific experimental conditions, propagation may happen in both directions.

However, some limitations should be acknowledged in our study. The number of *post mortem* samples was limited, and the ONE tissue was not represented across all Braak stages. In addition, the heterogeneous cellular composition and low neuronal content of nasal smears hindered precise biochemical quantification of tau. Notably, the quantification of p-tau181 was performed on a limited number of nasal smear samples, which should be taken into account when interpreting the statistical significance of the observed associations. Addressing these limitations in future work will allow a more complete characterization of tau pathology along the olfactory pathway.

4.3 | Olfactory deficits as indicators of tau pathology propagation

Olfactory function was evaluated using the standardized Sniffin' Sticks test. Compared to healthy controls, our results indicate that odor identification scores have declined significantly already at the preclinical stage of AD. In contrast, discrimination scores were impaired at the prodromal stage, while threshold scores only declined at the dementia stage. Our results suggest that functional impairments are closely linked to the development of tau pathology within the anatomical regions responsible for the respective olfactory functions. The decline in identification scores, which involves mainly cognitive function, was observed in preclinical A+T+ individuals, with no difference observed between A–T– healthy controls and preclinical A+T–, reinforcing that olfactory loss is more strongly linked to tau pathology rather than to amyloid burden.^{63–65} This is consistent with previous reports showing that odor identification is typically the first olfactory function to be affected in the early stages of AD, a function primarily controlled by central olfactory regions.^{66,67} In particular, several studies have shown that loss of odor identification ability is associated with the presence of NFTs in the EC and the CA1 region of the hippocampus, both central olfactory structures affected from the earliest stages of the disease.^{11,68,69} Furthermore, we observed an emerging correlation between threshold decline and the p-tau181/total tau ratio in nasal brushing, a measure reflecting neuroepithelial activity, providing novel evidence that peripheral olfactory function might also be associated with tau pathology.

Importantly, the temporal sequence of olfactory impairments parallels the anatomical progression of tau pathology inferred from the conclusions drawn from the analysis of OB and nasal smears. Interestingly, discrimination function, partially dependent on the OB,^{70,71} is altered at the prodromal stage. This alteration appears to correlate with NFT-like aggregates detected in the bulb at the same stage in the immunostaining performed on the OB samples, in agreement with a previous report of discrimination impairment at similar stages.⁵⁸

Beyond global scores, the analysis of individual odorants revealed selective impairments. Identification of orange scent was already reduced at the preclinical stage for T+ participants and continued to

decline in prodromal and dementia stages. Leather and anise identification declined from the prodromal stage. This selective pattern, which confirms previous work for early affected orange scent in preclinical AD,³² supports a central origin of early olfactory deficits rather than a diffuse peripheral loss, which would be expected to affect identification of all odors. The specific early vulnerability of certain odorants, such as orange, raises questions about whether distinct neural circuits or receptor populations are selectively involved in the earliest phases of tau pathology spread.

From a translational perspective, the identification of specific odors and olfactory domains that are impaired at defined disease stages opens the possibility of developing targeted olfactory tests as complementary biomarkers for AD. In future work, longitudinal studies should investigate whether individuals with lower baseline olfactory performance, particularly in early-affected odorants, show faster clinical progression, as suggested by previous reports.^{15,52,72} Such approaches, combined with comparative studies in other major neurodegenerative disorders, will be essential to determine the specificity and predictive value of olfactory testing for AD.

4.4 | Limitations and future directions

Several considerations should be noted. *Post mortem* analyses of OB at Braak stage II were limited to a single case, and future studies including samples across the full range of Braak stages will be important to better capture disease progression. For a fraction of *post mortem* OB samples, the cause of death was unknown. For smear analyses, age-matched comparisons could not be systematically performed given the number of participants recruited, although the mean age difference across groups was relatively small. Another limitation is that the z scores used for cognitive stratification (CN, prodromal, dementia) were not adjusted for age, sex, or education. However, z scores were derived from a single, homogeneous reference sample of 32 amyloid-negative individuals who remained cognitively stable over an 8-year follow-up,⁴⁵ rather than from heterogeneous external normative datasets. This biologically and clinically well-characterized reference sample likely improved the consistency and comparability of standardized cognitive scores across domains and tests. In addition, cognitive stratification was reviewed by neurologists and neuropsychologists, incorporating functional status and all available clinical information. Finally, analyses in the A+ CN group were conducted in a relatively small cohort ($n = 15$, 8 T- and 7 T+), which may reduce statistical power; nevertheless, these findings provide valuable preliminary observations that warrant replication in larger samples.

Finally, although patients with Lewy bodies disorders were excluded as rigorously as possible based on clinical criteria, the lack of sensitive *in vivo* diagnostic tools means that the presence of undetected Lewy body pathology cannot be entirely ruled out. Given its known impact on olfactory function,⁷³ this potential overlap may represent a limitation, as part of the observed olfactory deficits might not be exclusively attributable to AD pathology.

Overall, our results highlight the strong potential of olfactory testing for improving AD diagnosis and staging. Expanding odor panels linked to early misidentification may enhance diagnostic value. However, the absence of detectable tau aggregates in nasal smears from preclinical individuals suggests limited utility for early diagnosis. Future proteomic and transcriptomic analyses may help identify alternative early biomarkers in the ONE. Additionally, possible contributions of anterograde olfactory pathways to early OB involvement warrant further investigation, opening new perspectives for AD research and prevention.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. Author disclosures are available in the [supporting information](#).

ETHICS STATEMENT

All participants provided written informed consent prior to inclusion in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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RESEARCH ARTICLE OPEN ACCESS

Telomere Dysfunction and Proteostasis Decline Define Distinct Pathways of Cellular Senescence in the Human Respiratory Tract

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ABSTRACT

As the global population ages, cellular senescence contributes increasingly to the burden of age-related diseases. Hallmarks of this process include telomere shortening and loss of proteostasis, frequently linked to DNA damage-associated transcriptional stress. Although telomere dysfunction-induced foci (TIF) have been well documented in lungs from patients with idiopathic pulmonary fibrosis (IPF), their occurrence and role during physiological lung aging remain unclear. Analysis of senescence markers in lung tissue from organ donors aged 16–88 years showed a linear decline in telomere length with age; however, TIF frequency increased significantly in the airway epithelium only in individuals older than 75 years. Similarly, senescence markers such as p16 tended to rise with age but did not reach the levels observed in IPF lungs. To better delineate the early events driving senescence in the human respiratory epithelium and to expand the cohort size, we collected nasal epithelial cells by brushing from 213 healthy volunteers aged 2–97 years. As in the aging lung, telomere shortening was evident, yet TIF were rare and detected almost exclusively in individuals over 80 years of age. In contrast, indicators of impaired proteostasis, including increased senescence-associated β -galactosidase activity and lysosomal content, were apparent from the age of 40 in nasal epithelial cells and correlated with olfactory decline. Together, these findings suggest that telomere dysfunction is unlikely to be the primary driver of cellular senescence in the human respiratory tract, where proteotoxic stress may instead play a more prominent role.

Céline Coquette, Kamar Bouchoucha, and Manon Mahieu contributed equally.

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1 | Introduction

Aging is the primary global risk factor for disease and death, marked by a progressive loss of physiological function. In the human respiratory tract, aging increases susceptibility to lung diseases, impairs olfactory function, and heightens vulnerability to viral infections. The nasal epithelium serves as a crucial first line of defense against inhaled pathogens, allergens, and environmental irritants, and plays a central role in the pathogenesis of both acute and chronic airway disorders (Balazs et al. 2022). Aging of the upper respiratory tract is strongly associated with reduced olfactory capacity (Doty and Kamath 2014; Doty et al. 1984) and has recently been shown to alter nasal epithelial responses to SARS-CoV-2 infection (Woodall et al. 2024). Elucidating the molecular mechanisms that drive respiratory tract aging may pave the way for novel therapeutic strategies aimed at reducing the burden of these conditions.

The discovery of five core hallmarks of aging—genomic instability, telomere shortening, epigenetic changes, loss of proteostasis, and impaired macroautophagy (Lopez-Otin et al. 2023)—has significantly advanced our understanding of the aging process. However, a central challenge in aging research remains: identifying which of these hallmarks act as primary drivers of cellular aging. This question is further complicated by the fact that cellular senescence is heterogeneous, with aging hallmarks displaying organ-specific and time-dependent patterns (Cohn et al. 2023; Schaum et al. 2020), suggesting that there is no universal answer.

Although age-related telomere shortening is well documented in human tissues (Everaerts et al. 2018; Takubo et al. 2010), it is not sufficient on its own to induce telomere dysfunction, as defined by the formation of telomere dysfunction-induced foci (TIF) and the persistent activation of the DNA damage response, which ultimately results in irreversible cell cycle arrest and the onset of cellular senescence (Fumagalli et al. 2012; Hewitt et al. 2012; Rossiello et al. 2022). TIF have been observed in cells from individuals with telomere biology disorders (TBDs) and during replicative senescence in cultured fibroblasts (Rossiello et al. 2022). Lung cells from patients with pulmonary diseases such as idiopathic pulmonary fibrosis (IPF) also display an increased frequency of TIF (Birch et al. 2015; Schafer et al. 2017), and TIF have occasionally been reported in the context of physiological aging in the human lung (Birch et al. 2015; Schafer et al. 2017). However, whether TIF frequency consistently rises with age and contributes to normal physiological aging remains unclear.

During the past decade, proteotoxic stress has emerged as a potential central factor in cellular senescence induction (Ding et al. 2025; Kaushik and Cuervo 2015; Lopez-Otin et al. 2013, 2023). With age, misfolded and damaged proteins accumulate, in part due to increased reactive oxygen species (ROS) production. Combined with a decline in lysosomal function, this leads to elevated lysosomal content in aging cells evidenced by the heightened activity of senescence-associated β -galactosidase (SA- β -Gal) (Dimri et al. 1995). The age-related decline in macroautophagy efficiency further impairs the clearance of damaged organelles, such as mitochondria, exacerbating ROS levels and cellular damage. In vivo evidence for the role of defective autophagy in aging comes from studies showing that restoring

autophagy in aged mouse livers improves mitochondrial function and restores hepatic health (Zhang and Cuervo 2008). Additionally, recent research links proteostasis loss to DNA damage-induced transcriptional stress, resulting in widespread gene expression downregulation in aging cells (Gyenis et al. 2023; Stoeger et al. 2022).

In this cross-sectional study, we evaluated markers of cellular senescence in lung tissues from organ donors across a broad age range, as well as in freshly collected nasal brushing samples. Although telomere shortening was clearly evident in both tissue types, TIF abundance in the airway epithelium only significantly increased in donors aged 75 and older, reaching levels comparable to those found in IPF lungs. In contrast, nasal epithelial cells showed minimal age-related changes in TIF frequency. However, signs of proteostasis disruption, including elevated SA- β -Gal activity and increased lysosomal content, emerged as early as age 40 and were associated with olfactory decline. These findings suggest that although age-related telomere dysfunction in the lung epithelium may contribute to IPF pathogenesis, it is unlikely to serve as a universal trigger of cellular senescence. Rather, proteotoxic stress appears to play a more prominent role in driving age-associated tissue dysfunction in the upper respiratory tract.

2 | Methods

2.1 | Lung Samples

Unused or declined lungs for transplantation at University Hospital Leuven (Belgium) were obtained from a total of 20 donors (6 females and 14 males, 16–83 years) (Table S1). Collection of donor lungs for research was performed in accordance with Belgian legislation and approved by the local ethics committee (S59648, S61653). Lungs affected by emphysema were excluded. Because standardized in vivo chest CT scans were not available for these donors who lacked pre-mortem imaging suitable for quantitative emphysema assessment, we excluded emphysema using high-resolution ex vivo CT of inflated, frozen lung specimens. This approach enables the detection of macroscopic and regional parenchymal destruction characteristic of emphysema. Although microscopic, age-related alveolar simplification cannot be entirely ruled out, the absence of radiologic evidence of emphysema on ex vivo CT strongly supports the exclusion of emphysematous pathology in the analyzed specimens. Lungs with IPF ($n = 12$, 3 females and 9 males, 53–66 years) were obtained from patients undergoing lung transplantation at University Hospital Leuven (Belgium) (Table S1). IPF was diagnosed through dynamic multidisciplinary discussion according to ATS/ERS guidelines (Raghu et al. 2018). Written informed consent was obtained from all patients, and the study was approved by the local hospital ethical committee (S52174). Lungs were collected as previously described (Verleden et al. 2021). Briefly, lungs were air-inflated immediately after resection, snap-frozen in liquid nitrogen fumes, and subsequently sectioned transversally into 2-cm slices stored at -80°C . For immunohistochemistry, selected cores were formalin-fixed and paraffin-embedded (FFPE). Frozen tissue samples were incubated in 10% formalin for 24 h at room temperature before processing with a Leica TP1020 automated tissue processor (Leica Biosystems, Wetzlar,

Germany). Samples were then carefully oriented transversally and embedded in paraffin using a Leica Histocore Arcadia system. Additionally, peripheral wedge biopsies were collected from $n=13$ control donor lungs (7 females and 6 males, 28–88 years) prior to implantation (Table S1) (Van Slambrouck et al. 2025). Biopsies were taken from the lingula of the left upper lobe, in accordance with institutional ethical approval (S67052). The staple line was removed and four small pieces were promptly placed in cryotubes and frozen on dry ice before storage at -80°C .

2.2 | Nasal Brushing Sample Collection

Healthy volunteers ($n=213$, 117 females and 96 males, 2–97 years) were recruited from three independent clinical studies, all approved by the local ethics committee (Comité d’Ethique Hospitalo-Facultaire Saint-Luc–UCLouvain) under the following references: 2016/21MAR/121, 2021/10NOV/467, and 2022/07FEV/046 (Table S2). Individuals with TBD ($n=11$, 7 females and 4 males, 18–62 years) carrying pathogenic variants in *TERT*, *RTEL1*, or *TERC* were enrolled from study 2022/07FEV/046 (Table S2). Inclusion criteria for all participants were the absence of any upper respiratory tract infection within the three weeks prior to sampling and no history of chemotherapy or radiotherapy. Written informed consent was obtained from all participants, parents, and/or legal guardians. Assent was obtained from minors when appropriate. The studies were conducted in accordance with institutional ethical guidelines.

Nasal epithelial samples were collected by a trained physician using a standardized brushing technique. A sterile cytology brush was gently inserted into the lower half of each nostril, targeting the middle turbinate and the lower part of the nasal cavity below it. The brush was rotated to obtain epithelial cells, with one brushing performed per nostril. Both brushes were immediately immersed in 1 mL DMEM/F12 medium (11039021, Gibco, Thermo Fisher Scientific) supplemented with 10% FBS (SV30160.03, Cytiva) and 1% Penicillin/Streptomycin solution (PS-B, Capricorn Scientific). Cells were mechanically dissociated from the brushes and divided into three fractions, with cell numbers adjusted according to the requirements of each downstream application. The first fraction, used for immunofluorescence combined with FISH (IF-FISH), was applied to slides using a Cytospin centrifuge ($7\times g$, 5 min). The second fraction, intended for SA- β -Gal staining, was briefly centrifuged (30 s), the supernatant discarded, and the pellet fixed in 4% formaldehyde (F/1501/PB15, Fisher Scientific) in 1 $\times$ PBS for 5 min. After a second brief spin (30 s) and supernatant removal, the pellet was resuspended in 1 $\times$ PBS and applied to slides via Cytospin ($113\times g$, 10 min). The third fraction, reserved for qRT-PCR analyses, was pelleted (30 s spin) and stored at -80°C .

2.3 | Telomere Restriction Fragment (TRF) Length Analysis

TRF analyses were performed on genomic DNA extracted from lung tissue samples, as described previously (Episkopou et al. 2019), using 5 μg of RNase-treated genomic DNA digested with 20 U *HinfI* (R0155S, NEB) and 20 U *RsaI* (R0167L, NEB) and

a [γ - ^{32}P] ATP (BLU502A250UC, Revvity)-labeled (TAACCC)₄ probe (Eurogentec). Smart Ladder (MW-1700-10, Eurogentec) was run together with the samples, stained with SYBR Safe DNA Gel Stain (S33102, Thermo Fisher), and imaged with a ruler. The blots were exposed to a phosphor screen, scanned on Phosphorimager (GE Healthcare), and quantified using the online available WALTER (Web-based Analyzer of the Length of Telomeres) software (<https://www.celeec.eu/chromatin-molecular-complexes/rg51/tab?tabId=125#WALTER>).

2.4 | Immunofluorescence and Telomeric FISH

The protocol described previously (Episkopou et al. 2019) was adapted for TIF detection on frozen tissue sections. First, 5 μm -thick sections were briefly incubated in cold acetone for 5 min at room temperature (RT), then washed with 1 $\times$ PBS before fixation in 4% formaldehyde in 1 $\times$ PBS for 10 min at RT under gentle agitation. After an additional wash with 1 $\times$ PBS for 5 min at RT, samples were heated in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven (450 W), incubated in permeabilization buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100) for 1 h at 37°C , and then washed three times for 3 min each in 0.1% Tween-20 in 1 $\times$ PBS (PBS-T). Following these steps, tissue sections were either subjected to immunofluorescence (IF) using anti-TRF2 (1:2000) to label telomeres and anti- γ -H2AX (1:1000) to detect DNA damage, or first processed for telomeric FISH and subsequently stained by IF with γ -H2AX (1:1000) and pro-SPC (1:1000) to identify AT2 cells. In the first case, tissue sections were first incubated for 45 min at RT in 400 μL of blocking solution (2% normal goat serum (S26-M, Sigma-Aldrich), 0.2% BSA (A7030, Sigma-Aldrich) and 0.1% Triton X-100 in 1 $\times$ PBS) before adding the primary antibodies (Table S3) diluted in the same blocking solution and incubated overnight at 4°C . After three 3-min washes at 45°C in PBS-T, samples were incubated for 40 min at 45°C with secondary antibodies (Table S3) diluted 1:400 in blocking solution. Samples were then washed three times for 3 min each in PBS-T at 45°C , followed by three final washes in 1 $\times$ PBS at RT. After air drying, samples were mounted with fluorescence mounting medium (S302380-2, Agilent Technologies) containing 0.1 $\mu\text{g}/\text{mL}$ DAPI (D9542, Sigma-Aldrich). When telomeric FISH was performed, tissue sections were first incubated with RNase A solution (0.1 mg/mL, R4875, Sigma-Aldrich) in 2 $\times$ SSC for 1 h at 37°C . After three washes of 3 min each in PBS-T, slides were dehydrated in successive ethanol baths (70%–80%–90%–100%), 3 min each. Slides were air-dried before incubation with the FISH hybridization solution (160 nM G-rich red LNA (5'-TYE563/GGGT+TAGGG+T+TAG+GGTTAGGG+T+TAGGG+T+TAGGG+TTA/3'-TYE563, 339414, Qiagen) telomeric probe, 50% deionized formamide (P040.2, Carl Roth), 2 $\times$ SSC, 1 $\times$ Blocking reagent (11096176001, Roche)). The first incubation was performed at 83°C for 10 min, then slides were kept at 37°C in the dark for 2 h. Slides were then washed two times for 15 min in 50% formamide, 2 $\times$ SSC, 20 mM Tris-HCl pH 7.4, and three times for 5 min in 150 mM NaCl, 0.05% Tween-20, 50 mM Tris-HCl pH 7.4. Finally, slides were dehydrated as above and air-dried before processing with IF as described above.

For TIF detection in nasal brushing samples, we used the combination of telomeric FISH and IF against γ -H2AX. Nasal

epithelial cells, spread on slides, were first incubated for 5 min in permeabilization buffer, then fixed for 15 min in 4% formaldehyde in 1× PBS, and subsequently re-incubated in permeabilization buffer for 10 min. IF steps were performed as described above, using antibodies against γ -H2AX (1:1000) and anti-mouse Alexa Fluor 647 as secondary antibodies (1:400) (Table S3). After washes, cells were fixed again for 2 min with 4% formaldehyde in 1× PBS, washed three times for 3 min with 1× PBS, and incubated with the RNase A solution for 1 h at 37°C. Slides were washed again three times for 3 min with 2× SSC before incubation in permeabilization buffer for 10 min at RT. After a last fixation for 2 min with 4% formaldehyde in 1× PBS, slides were dehydrated in successive ethanol baths (70%–80%–90%–100%), 2 min each. Slides were air-dried before incubation with the FISH hybridization solution. The first incubation was performed at 83°C for 3 min, then slides were cooled down to RT for 2 h. Finally, slides were washed two times for 15 min in 50% formamide, 2× SSC, 20 mM Tris–HCl pH 7.4, and then three times for 5 min in 150 mM NaCl, 0.05% Tween-20, 50 mM Tris–HCl pH 7.4. Slides were dehydrated as above and air-dried before mounting with fluorescence mounting medium.

For detection of lysosomes and CD68+ cells in nasal brushing samples, spread cells were permeabilized for 30 min at RT in Tris–HCl buffer with 0.5% Triton X-100, rinsed with 1× PBS, and blocked for 1 h at RT in 1× PBS containing 0.1% Triton X-100, 0.21% BSA, and 2.1% normal goat serum. Slides were then incubated overnight at 4°C with primary antibodies (Table S3) diluted in blocking solution: LAMP2 (1:200), β -catenin (1:200), or CD68 (1:200). Samples were incubated three times for 3 min each in PBS-T at 45°C before incubation for 40 min at RT with secondary antibodies diluted in blocking solution: anti-mouse Alexa Fluor 647 and anti-rabbit Alexa Fluor 488 (1:1000). This was followed by another series of three 3-min washes with PBS-T at 45°C, and a final series of three 3-min washes with 1× PBS at RT. Slides were mounted as described above. Images were acquired using a Cell Observer Spinning Disc confocal microscope (Zeiss) with either a 40× or a 100× oil immersion objective. Image processing was performed using ImageJ (imagej.nih.gov).

2.5 | p16 Immunohistochemistry on Lung Sections

p16 immunohistochemistry (IHC) was performed on 5 μ m-thick FFPE lung sections according to the manufacturer's instructions using the mouse E6H4 anti-p16 monoclonal antibody and the VENTANA detection kit (705-4793, Roche) on a BenchMark ULTRA automated slide stainer. Image acquisition and processing was performed as described above. After IHC, IPF lung sections were stained with Alcian Blue (A3157-10G, Sigma-Aldrich) to detect fibroblastic foci.

2.6 | RNA Extraction and qRT-PCR

For lung samples, total RNA was extracted from ten 10 μ m-thick cryosections of frozen tissue using TriPure reagent (11667165001, Roche), following the protocol described previously (Episkopou et al. 2019). For nasal brushing samples, total

RNA was extracted from cell pellets using the RNeasy Plus kit (74006, Qiagen). Pellets were resuspended in 350 μ L RLT buffer containing 1% β -mercaptoethanol, followed by the addition of 350 μ L 70% ethanol. The suspension was transferred to the spin column and centrifuged for 30 s at 8000×g at RT. Bound RNA was washed with 350 μ L RW1 buffer and centrifuged for 30 s at 8000×g. On-column DNase digestion (2.7 U/ μ L; 79,256, Qiagen) was performed to remove genomic DNA, followed by an additional RW1 wash. Two washes with 500 μ L RPE buffer were carried out, each followed by centrifugation (30 s at 8000×g, then 2 min at 8000×g), and a final 1 min centrifugation at maximum speed. RNA was eluted by incubating 25 μ L RNase-free water (RF) on the membrane for 20 min, followed by centrifugation for 30 s at 8000×g. cDNA synthesis and qRT-PCR analyses were performed as described previously (Episkopou et al. 2019) using primers described in Table S4.

2.7 | SA- β -Gal Activity

For lung samples, the SA- β -Gal activity assay was performed at pH 5.6 on 16 μ m-thick sections as described previously (Froidure et al. 2020). Briefly, after fixation with 0.2% formaldehyde for 10 min, sections were rinsed with 1× PBS before overnight incubation at 37°C in staining solution (40 mM citric acid/Na phosphate, pH 5.6, 5 mM $[K_4[Fe(CN)_6] \cdot 3H_2O]$ (P9387, Sigma-Aldrich), 5 mM $K_4[Fe(CN)_6]$ (702,587, Sigma-Aldrich), 150 mM NaCl, 2 mM $MgCl_2$, 1 mg/mL X-gal (B4252, Sigma-Aldrich)). The next day, slides were scanned using the Mirax Midi Imaging system (Zeiss). Images were analyzed with QuPath (qupath.github.io). For nasal brushing samples, spread cells were first rinsed with 1× PBS before incubation for 18 h at 37°C in the staining solution at pH 6. A positive control was performed at pH 4. After rinsing with 1× PBS and mounting with 90% glycerol in distilled water, images were acquired using an EVOS XL Core microscope (Invitrogen, Thermo Fisher Scientific) in brightfield mode to count blue-stained cells and in phase-contrast mode to count total cells, using a 40× objective. Results at pH 6 were excluded if the percentage of blue cells at pH 4 was below 85%.

2.8 | Single Telomere Length Analysis (STELA)

STELA analysis was performed as described previously (Baird et al. 2003). Briefly, genomic DNA was extracted using QIAamp DNA Micro Kit (56304, Qiagen) and eluted in 35 μ L 10 mM Tris–HCl (pH 8) and 1 μ L of Telorette2 linker (10 μ M) added to the DNA solution. This was diluted to 500 pg/ μ L. One μ L of this diluted DNA/Telorette2 solution were subjected to PCR in a 10 μ L reaction containing 0.5 μ M 17p telomere-specific primer (17pse-q1rev: 5'-GAATCCACGGATTGCTTTGTGTAC-3'), 0.5 μ M Teltail primer and 0.5 U of a 10:1 mixture of Taq (Thermo Fisher Scientific) and Pwo polymerase (11644955001, Roche). PCR amplicons were resolved by 0.5% TAE agarose gel electrophoresis and were detected by Southern hybridization with a random-primed α -³²P-labeled (Hartmann Analytic GmbH) TTAGGG repeat probe together with a probe to detect 1 kb (Stratagene) and 2.5 kb (170–8205, BioRad) molecular weight markers. The blots were exposed to a phosphor screen, scanned on Typhoon FLA 9500 phosphorimager (GE Healthcare) and analyzed using ImageQuant TL (GE Healthcare).

2.9 | Olfactory Tests

Healthy volunteers ($n = 72$, 42 females and 30 males, 18–97 years) were recruited from two independent clinical studies, all approved by the local ethics committee (Comité d’Ethique Hospitalo-Facultaire Saint-Luc-ULouvain) under the following references: 2016/21MAR/121 and 2022/07FEV/046 (Table S2). Individuals with TBD ($n = 10$, 6 females and 4 males, 18–62 years) carrying pathogenic variants in *TERT*, *RTEL1*, or *TERC* were enrolled from study 2022/07FEV/046 (Table S2). Written informed consent was obtained from all participants, parents, and/or legal guardians. Assent was obtained from minors when appropriate. The studies were conducted in accordance with institutional ethical guidelines.

Olfactory abilities were measured with the Sniffin’ Sticks test (200112, MediSense) (Hummel et al. 2017). The test yields three separate scores: odor threshold (T), discrimination (D), and identification (I). The stimuli are presented via felt-tip pens filled with odor solutions, which are held just below the nostrils and gently moved past them for several seconds. The threshold task involves phenylethanol, serially diluted across 16 concentrations. In each trial, participants are given three pens, only one of which contains the odor. Using a forced-choice procedure, they attempt to detect the correct pen. A staircase rule was applied, where performance determined whether weaker or stronger concentrations were presented. After seven reversals, the threshold value was computed as the mean of the last four reversals, producing a score between 1 and 16. Discrimination is assessed with 16 sets of three pens, where two pens contain the same odor and the third differs. With eyes closed, participants indicate the odd stimulus. The number of correct responses provides the discrimination score (0–16). For identification, 16 familiar odors—commonly recognized by most healthy individuals—are presented individually. Participants choose the correct label from four options read aloud by the examiner, yielding an identification score between 0 and 16. The overall measure of olfactory performance, the TDI score, is obtained by summing the three subscores, with a maximum of 48 points.

2.10 | Statistical Analyses

All analyses were performed using the GraphPad Prism software. Graphs were also obtained with GraphPad Prism. The statistical tests that were applied are detailed in the figure legends.

3 | Results

3.1 | TIF Increase Occurs Late in the Aging Human Airway Epithelium

To monitor TIF formation in the aging human lung, we used tissue samples from organ donors aged 16–88 (Table S1). Consistent with previous findings (Lee et al. 2021; Van Slambrouck et al. 2025), we observed a linear, age-related decrease in telomere length (TL) (Figure 1A,B). In our lung tissue cohort, the age-dependent TL attrition was of about 42 ± 5 bp per year, in line with the 20–60 bp range previously proposed for yearly rates of TL reduction in human tissues (Takubo et al. 2010).

TIF are identified by the co-localization of DNA damage markers with telomeres (d’Adda di Fagagna et al. 2003). Here, TIF were assessed by immunofluorescence-based co-detection of telomeres (TRF2) and γ -H2AX DNA damage marker (Figures 1C and S1A). Although the number of lung tissue samples analyzed was limited, we observed that the average number of TIF per nucleus in airway epithelial cells was significantly higher in tissues from donors aged 75–88 ($n = 8$) compared to those aged 16–47 ($n = 7$), with a median of 3.5 TIF per nucleus in the oldest group (Figure 1D). In contrast, this increase was not significant in the other cell types of the same lung samples (Figure 1C,E). In the oldest airway epithelial tissues, nuclei frequently contained more than one TIF (Figure 1F). In line with the elevated TIF levels in the aging airway epithelium, p16 staining was notably more intense in this region (Figure 1G). These findings were further supported by a trend toward elevated *p16* mRNA expression in lung tissue extracts from aged individuals (Figure S1B,C).

Aging of the lung epithelium represents a major risk factor for IPF, a fatal disease characterized by cellular senescence and progressive lung scarring driven by excessive extracellular matrix deposition, with a prevalence that increases markedly after the age of 75 (Hewitt et al. 2025; Schneider et al. 2021). Our analyses revealed an increase in hallmarks of airway epithelial senescence in individuals older than 75 years. We therefore next compared age-associated airway epithelial senescence in the lung with the senescence profile observed in IPF lungs. To address this, we compared TIF in lung samples from IPF patients aged 53–66 ($n = 12$), age-matched controls (aged 56–63, $n = 8$), and older controls aged 75 and above ($n = 8$) (Table S1). Although the number of IPF lung samples analyzed was limited, TIF abundance in the airway epithelial tissue from IPF lungs (median of 2.2 TIF/nucleus) was approximately fivefold higher than in age-matched controls (median of 0.4 TIF/nucleus) (Figures 2A,B and S2A). This level, however, was comparable to that observed in control lungs from donors aged 75 and above (Figures 2A,B and S2A). Similar results were obtained when comparing the percentage of cells containing at least two TIF per nucleus (Figure 2C). Previous work reported shorter telomere length in Alveolar type 2 (AT2) cells of IPF patients (Snetselaar et al. 2017). As AT2 cell dysfunction may be an important driver of IPF (Katzen and Beers 2020), we next tested whether AT2 alveolar cells may show distinct TIF positivity in both aged and IPF lungs. However, using combined telomeric FISH and immunofluorescence for γ H2AX and pro-SPC to specifically identify AT2 cells in IPF lungs, we found that the frequency of TIF-positive cells was not significantly different between AT2 and non-AT2 alveolar cells, nor between AT2 alveolar cells and airway epithelial cells (Figure S2B,C). Likewise, the frequency of TIF-positive AT2 alveolar cells did not differ significantly between aged donors and donors with IPF, consistent with the results obtained in airway epithelial cells (Figure S2B,C). These results align with previous findings showing that, in IPF lungs, both bronchial epithelial cells and alveolar compartments exhibit comparable positivity for senescence markers (Schafer et al. 2017).

The “second hit” hypothesis posits that external insults to an already compromised, aging lung epithelium contribute to the onset of IPF (Hewitt et al. 2025). Our findings indicate that TIF may be involved in establishing this age-related epithelial susceptibility. However, since TIF levels were comparable

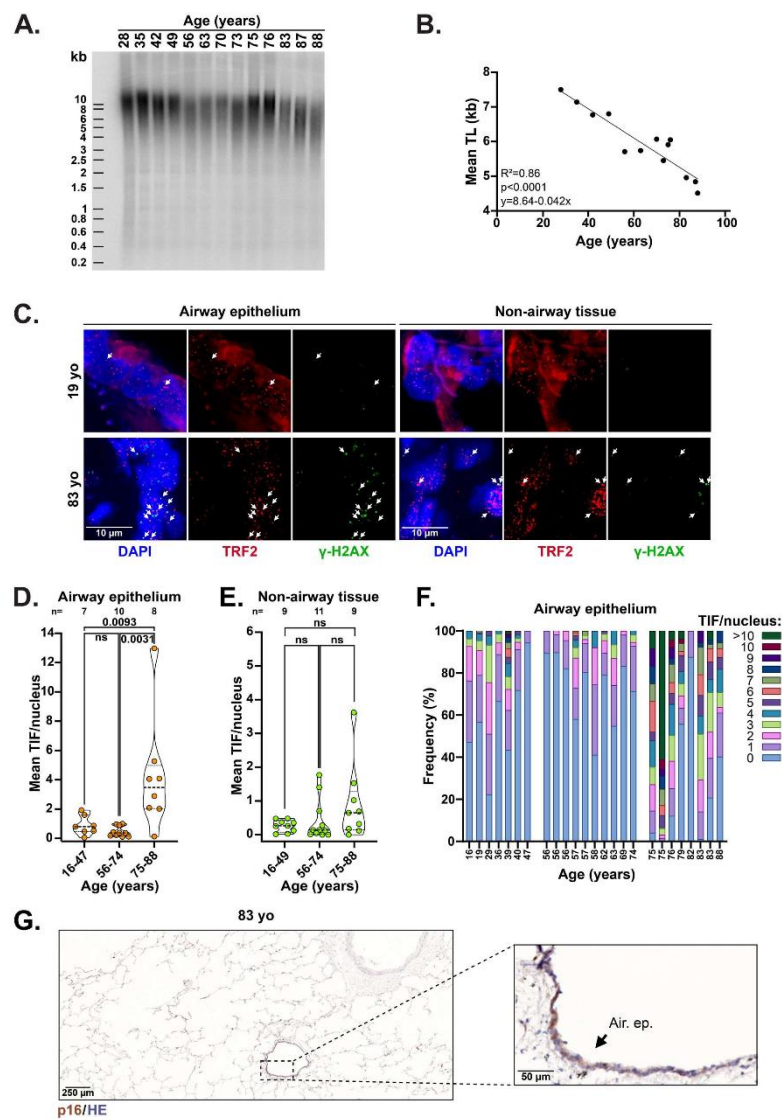


FIGURE 1 | Legend on next page.

FIGURE 1 | TIF increase occurs late in the aging human airway epithelium. (A) TRF analysis of TL in lung tissues from control donors (Ctrl) aged 28–88 years. Ladder (kb). (B) Linear regression analysis corresponding to panel A. (C) Representative images of TIF analysis in the airway epithelium (left) and non-airway tissues (right) of control lungs from 19- and 83-year-old donors. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bar: 10 μ m. (D) Mean number of TIF per nucleus in the airway epithelium of donors across the specified age ranges. On average, around 75 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Mann–Whitney test. (E) As in panel D, but for the non-airway tissues. On average, around 100 nuclei were analyzed per sample. Mann–Whitney test. (F) Distribution of nuclei by the number of TIF per nucleus in the airway epithelium of donors at the indicated ages. (G) Representative images of p16 staining by immunohistochemistry in lung sample from a 83 years old donor. Air. Ep., airway epithelium; HE, hematoxylin–eosin. Scale bars: 250 and 50 μ m.

between lungs from IPF donors and age-matched controls (≥ 75 years) with markedly different degrees of cellular senescence, TIF alone appears insufficient to drive the extensive senescence observed in IPF. This is supported by significantly elevated *p16* mRNA levels in IPF lungs (Figure 2F), and strong p16 protein staining in epithelial cells near fibrotic regions, marked by Alcian blue, consistent with previous studies (Minagawa et al. 2011; Schafer et al. 2017) (Figures 2G and S2D). The heightened senescence observed in IPF is further supported by a concurrent increase in SA- β -Gal activity within the epithelial layer (Figure 2H).

Collectively, these findings indicate that the rise in TIF frequency occurs only at a very advanced age, specifically within the lung epithelial cells of donors aged 75 and older, and support a potential causal role for TIF in IPF pathogenesis. Although additional IPF lung samples will be required to confirm these observations, it is likely that further external insults are required to initiate the pronounced cellular senescence observed in the IPF lung epithelium, characterized by elevated p16 expression and SA- β -Gal activity.

3.2 | Telomere Shortening Without Major TIF Induction in Aging Nasal Epithelial Cells

Cellular aging progresses along distinct, organ-specific trajectories and exhibits temporal variability (Schaum et al. 2020). To investigate whether senescence markers in the upper respiratory tract mirror those observed in the aging lung epithelium, we developed a minimally invasive nasal brushing method to collect epithelial cells from healthy donors ranging in age from 2 to 97 years (Table S2 and Figure S3). First, we assessed whether TL decreases with age in nasal epithelial cells. Using STELA analysis (Baird et al. 2003) on samples from young ($n = 3$) and older ($n = 5$) donors, we estimated an age-related TL attrition rate of approximately 64 ± 8 base pairs per year (Figure 3A,B, black). This value is comparable to the 59–60 bp/year telomere shortening rate observed in the colon and the esophagus, with high regenerative activity (Takubo et al. 2010). Nasal brushing samples from two individuals with telomere biology disorders (TBD), carrying mutations in either the *TERC* (age 56) or *TERT* (age 29) subunit of telomerase, revealed markedly short telomeres, comparable to the average TL observed in nasal epithelial cells from control individuals aged 75 and older (Figure 3A,B). Collectively, these findings support the utility of nasal epithelial cells as a valid model for studying telomere dynamics during aging.

Analysis of TIF formation in nasal epithelial cells revealed a modest age-associated increase in TIF levels (Figure 3C,D). Despite this trend, the median number of TIF per nucleus remained very low, rising only slightly from 0 to 0.04 across age groups (Figure 3D), indicating a generally low TIF abundance in this tissue. Nasal epithelial cells from TBD patients aged 19–62 displayed a higher TIF burden compared to age-matched controls, but levels were similar to those seen in the older control group aged 80 and above (Figure 3E–G). This supports the notion of overall low TIF abundance in nasal epithelium. Additionally, our finding that the average number of γ -H2AX foci in samples from older individuals did not significantly increase (Figure S4) argues against the possibility of a bias in TIF detection resulting from telomeres being too short to be visualized by FISH. In the olfactory neuroepithelium—located near the nasal epithelium—and sampled from two deceased donors aged 83 and 94 years, TIF levels were also extremely low (median of 0) (Figure 3H,I). These results contrast with the increased TIF abundance observed in the aging lung epithelium, highlighting the previously reported variability in age-dependent TIF (Jeyapalan et al. 2007).

These findings indicate that, although TL decreases with age, nasal epithelial cells from healthy individuals show minimal accumulation of TIF.

3.3 | Early Proteostasis Disruption in Nasal Epithelial Cells

As no single marker can definitively identify senescent cells (Gorgoulis et al. 2019), and given the minimal accumulation of TIF observed in aged nasal epithelial cells, we proceeded to examine additional senescence markers in the nasal brushing samples.

The colorimetric assay for SA- β -Gal activity (Dimri et al. 1995), which reflects the elevated lysosomal content characteristic of senescent cells undergoing proteotoxic stress, remains the most widely used approach for identifying senescent cells both in vitro and in vivo (Dimri and Dimri 2024). Using this method, we found that the proportion of SA- β -Gal-positive cells (SA- β -Gal+) increases with age, showing a significant rise in individuals aged 40 and above, followed by a plateau around the age of 60 (Figure 4A). In the oldest age group—individuals over 80 years—approximately 30% of the collected cells were SA- β -Gal+ (Figure 4A). Notably, this increase in SA- β -Gal positivity occurred well before the modest rise in TIF frequency observed

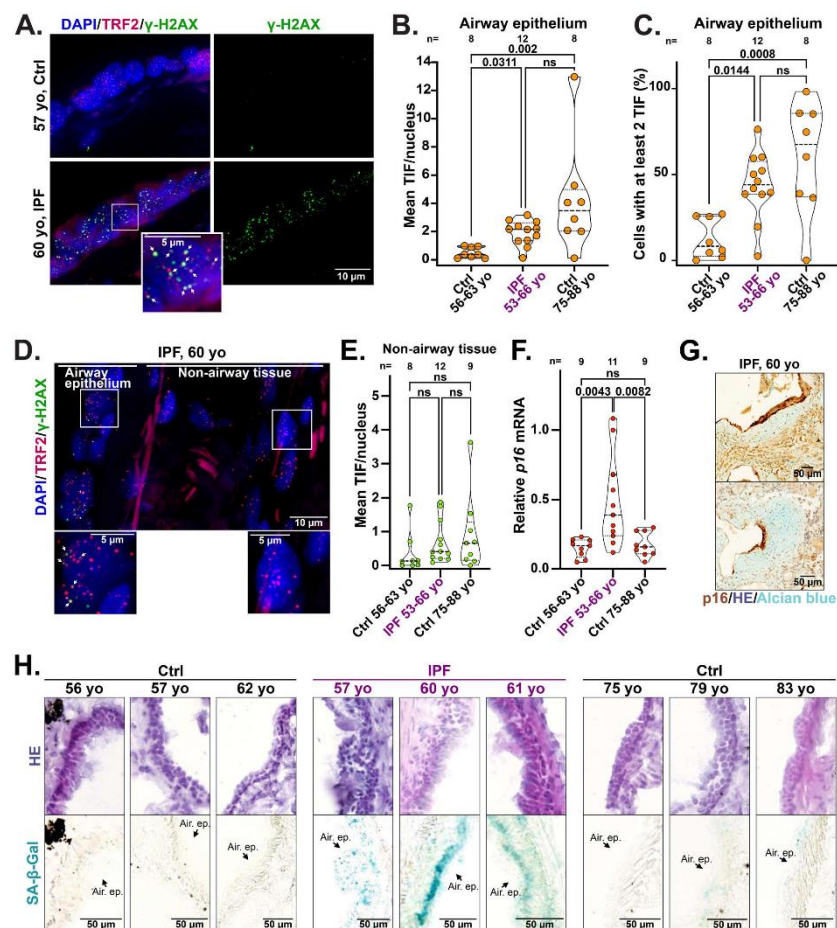


FIGURE 2 | Legend on next page.

in the oldest individuals (Figure 4A), indicating that SA-β-Gal expression does not result from TIF induction.

One limitation of using SA-β-Gal as a senescence marker is its tendency to also stain macrophages (Sharpless and Sherr 2015). To rule out the possibility that the observed increase in SA-β-Gal positivity in nasal brushing samples was due to macrophages, we measured *CD68* mRNA levels, a recognized macrophage marker. Despite the significant elevation in SA-β-Gal+ cells,

there was no statistically significant difference in *CD68* mRNA expression between the 0-40 and 40-100 years age groups (Figure 4B). This was confirmed by staining two independent nasal brushing samples, which revealed SA-β-Gal positivity clearly distinct from the proportion of CD68-positive cells (Figure 4C).

Earlier studies proposed that elevated SA-β-Gal activity in senescent fibroblasts reflects increased lysosomal content and

FIGURE 2 | Premature TIF and senescence in IPF airway epithelium. (A) Representative images of TIF analysis in the airway epithelium from a 57-year-old control donor (Ctrl) and a 60-year-old donor with IPF. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bars: 5 and 10 μ m. (B) Mean number of TIF per nucleus in the airway epithelium of Ctrl or IPF lungs across the specified age ranges as indicated on the graph. On average, around 90 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal–Wallis test. ns: Not significant. (C) Percentage of nuclei showing at least 2 TIF in the airway epithelium of Ctrl or IPF lungs across the specified age ranges as indicated on the graph. Violin plots showing median, first and third quartile. Ordinary one-way ANOVA test. (D) As in panel A, but including both airway epithelium and non-airway tissues of the IPF lung. Scale bars: 5 and 10 μ m. (E) Mean number of TIF per nucleus in the non-airway tissues of Ctrl or IPF lungs across the specified age ranges as indicated on the graph. On average, around 100 nuclei were analyzed per sample. Kruskal–Wallis test. ns: Not significant. (F) qRT-PCR analysis of *p16* expression in lung tissue samples from age-matched Ctrl and IPF donors, as well as Ctrl donors aged 75 and older. *p16* mRNA levels were normalized to *ACTB* mRNA and to one IPF lung sample used as control. Ordinary one-way ANOVA test. (G) *p16* staining by immunohistochemistry and Alcian blue staining of fibrotic area in lung sample from a 60-year-old donor with IPF. HE, Hematoxylin–Eosin. Scale bar: 50 μ m. (H) Representative images of SA- β -Gal activity staining in the airway epithelium of Ctrl or IPF lung samples from donors at the indicated age. Hematoxylin–Eosin (HE) staining is shown above. Scale bars: 50 μ m.

accumulation of degradative autolysosomes (Gerland et al. 2003). Accordingly, the rise in SA- β -Gal positivity in nasal epithelial cells around age 40 may indicate an early impairment in proteostasis. To strengthen this observation, we employed an orthogonal method by staining samples with an antibody against LAMP2, lysosomal-associated membrane protein, to assess lysosome enlargement. Cells were considered LAMP2+ if they showed intense cytoplasmic staining (Figure 4D). Consistent with SA- β -Gal staining and prior findings of increased LAMP2 expression in senescent melanoma cells (Rovira et al. 2022), the proportion of LAMP2+ nasal epithelial cells rose significantly with donor age and showed a strong correlation with SA- β -Gal positivity (Figure 4E).

Finally, we examined whether the increased SA- β -Gal positivity in aging nasal epithelial cells was linked to elevated expression of the senescence markers *p21* or *p16*. While *p21* mRNA showed a moderate correlation with SA- β -Gal positivity (Figure 4F), *p16* expression levels did not correlate with SA- β -Gal positivity in nasal epithelial cells (Figure 4G).

Together, these results point to an association between aging of nasal epithelial cells and proteostatic stress.

3.4 | SA- β -Gal Activity in Nasal Epithelial Cells Negatively Correlates With Olfactory Function

Olfactory function gradually declines beginning in early adulthood, with the most marked reduction observed in odor threshold sensitivity, as opposed to odor discrimination or identification (Oleszkiewicz et al. 2019). Odor discrimination and identification rely on both the olfactory nasal epithelium and central brain regions such as the entorhinal cortex and hippocampus to process the information (Kjelvik et al. 2012). Conversely, odor threshold is more directly influenced by olfactory nasal epithelium function (Hummel et al. 2017). To investigate whether aging of the olfactory nasal epithelium contributes to olfactory decline, we assessed odor threshold in healthy individuals aged 18 to 97. Using the Sniffin' Sticks test, we found that declining threshold scores with age were associated with increased SA- β -Gal positivity in nasal epithelial cells (Figure 5A,B), suggesting a potential link between proteostasis

loss in these aging cells and olfactory decline. Since the olfactory epithelium supports the maintenance and function of olfactory neurons, its aging is also likely to impair overall olfactory performance. In line with this, the total TDI score showed a negative correlation with SA- β -Gal positivity in nasal epithelial cells (Figure 5C). Interestingly, TBD patients did not exhibit any premature olfactory deficit (Figure 5D), further supporting the idea that telomere dysfunction is not a driver of aging in the olfactory nasal epithelium.

Taken together, these findings indicate that impaired proteostasis in the aging olfactory nasal epithelium contributes to the age-related decline in olfactory function.

4 | Discussion

In patients with TBD, telomere dysfunction is a well-established and widely accepted contributor to accelerated cellular and tissue aging, as well as the early onset of age-related diseases (Revy et al. 2023; Rossiello et al. 2022). In contrast, during normal aging, only low levels of damaged telomeres have been reported in various tissues of aging baboons, including skin, hippocampal neurons, and liver hepatocytes (Fumagalli et al. 2012; Herbig et al. 2006; Jeyapalan et al. 2007), as well as in skin biopsies from elderly human donors (Vitorelli et al. 2019). In both baboon and human tissues, the average TIF burden per nucleus remains modest compared to the elevated levels observed during replicative senescence in vitro (Jeyapalan et al. 2007; Vitorelli et al. 2019). While early studies in telomerase-deficient mice indicated that just one or a few critically short telomeres could be sufficient to trigger cellular senescence in vivo (Hemann et al. 2001), the extent to which TIF contributes to physiological aging in primates remains unresolved. Given the heterogeneity of cellular senescence (Cohn et al. 2023), it is also likely that the contribution of TIF to senescence is not universal across cell types or tissues.

Patients with TBD exhibit a strong predisposition to early-onset IPF, supporting a causal link between telomere dysfunction in the lung epithelium and IPF pathogenesis (Revy et al. 2023; Rossiello et al. 2022). In contrast, among individuals without TBD, IPF prevalence rises sharply only after the age of 75

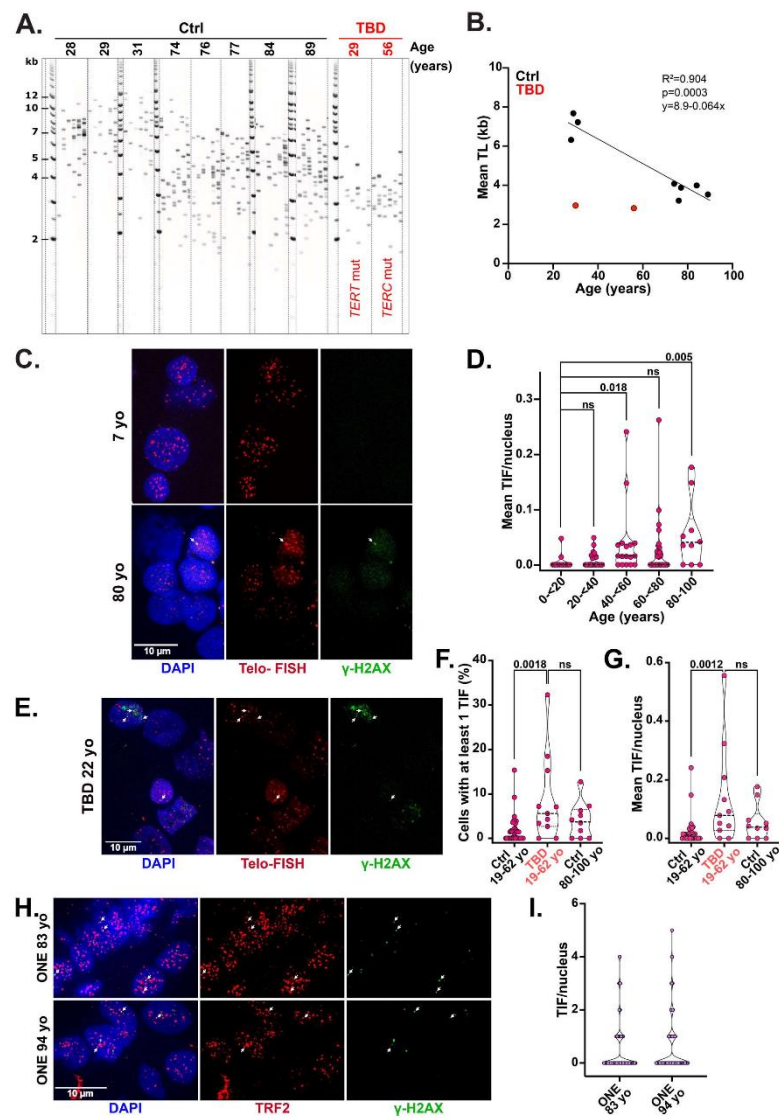


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FIGURE 3 | Telomere shortening without major TIF induction in aging nasal epithelial cells. (A) STELA analysis of TL in nasal brushing samples from donors without (Ctrl) or with telomere biology disorder (TBD). The age of the donors is indicated. Ladder (kb). (B) Linear regression analysis corresponding to panel A using nasal brushing samples from the Ctrl donors (black dots). Red dots represent TBD donors. (C) Representative images of TIF analysis in nasal brushing samples from Ctrl donors aged 7 and 80 years. Telomeres are detected by FISH with a telomeric probe (Telo-FISH, red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bar: 10 μ m. (D) Mean number of TIF per nucleus in the nasal brushing samples of Ctrl donors across the specified age ranges. On average, around 65 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal–Wallis tests. ns: Not significant. (E) As in panel C, but in the nasal brushing sample from a 22-year-old donor with TBD. Arrows indicate TIF. Scale bar: 10 μ m. (F) Percentage of nuclei showing at least one TIF per nucleus based on the analysis described in panel F. Violin plots showing median, first and third quartile. Kruskal–Wallis tests. ns: Not significant. (G) Mean number of TIF per nucleus in the nasal brushing samples from Ctrl and TBD donors as indicated on the graph. On average, around 80 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal–Wallis tests. ns: Not significant. (H) Representative images of TIF analysis in the ONE tissue of two donors aged 83 and 94 years. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Arrows indicate TIF. Scale bar: 10 μ m. (I) Number of TIF per nucleus in the ONE samples from panel H. A total of 85 and 101 nuclei were counted per sample, respectively.

(Hewitt et al. 2025; Schneider et al. 2021). Whether this is associated with TIF accumulation in the lung epithelium remains unclear, as most studies to date have focused on lung tissues from individuals younger than 80 years old. In this study, we show that TIF frequency in the airway epithelium rises significantly in donors aged 75 and older, reaching levels comparable to those observed in IPF lungs. Although we acknowledge that the limited number of lung samples analyzed represents a limitation of our study, these findings suggest a potential causal link between TIF accumulation and the development of IPF during physiological aging. The “second hit” hypothesis proposes that IPF arises from additional exogenous insults acting on an already vulnerable, aging lung epithelium (Hewitt et al. 2025). It is therefore plausible that TIF induction contributes to this vulnerability, and that further environmental exposures, such as cigarette smoke, intensify cellular senescence and drive fibrosis. Consistent with our previous findings showing that short telomeres elevate the risk of severe COVID-19 (Froidure et al. 2020), the late-life increase in TIF within the lung epithelium may also possibly contribute to the heightened vulnerability to COVID-19-related mortality, which is reported to be approximately 50 times higher in individuals aged 80 and above compared to those aged 18–39 (Williamson et al. 2020).

Age-related alterations in the nasal epithelium have been proposed to contribute to olfactory decline (Doty and Kamath 2014; Kondo et al. 2020; Mobley et al. 2014). Although the decline in odor identification function may be partly attributed to broader age-related deterioration in central nervous system functions, which we did not address in this study, severe but transient damage to the olfactory epithelium, independent of injury to the central nervous system, has been linked to anosmia, as observed during SARS-CoV-2 infection (Bryche et al. 2020). Similarly, cumulative environmental insults to the olfactory epithelium have been proposed as a contributing factor to age-related olfactory decline (Attems et al. 2015). While the exact mechanisms by which epithelial damage contributes to age-associated olfactory loss remain unclear, they may involve functional impairments such as reduced ciliary beat frequency and prolonged nasal mucociliary clearance, both of which have been reported in

individuals over the age of 40 (Ho et al. 2001). Aging of the upper respiratory tract, however, has been relatively understudied, and whether TIF play any role in this part of the respiratory tract remains unexplored. Our findings suggest that TIF are unlikely to be the primary drivers of age-related senescence in nasal epithelial cells. Instead, we propose that this nasal epithelium senescence results from a gradual loss of proteostasis beginning after the age of 40. This is consistent with recent findings suggesting a widespread breakdown of proteostasis in aging human tissues, with a notable acceleration in aging processes typically occurring around the age of 50 (Ding et al. 2025). Proteostasis, the maintenance of a balanced system for protein synthesis, folding, trafficking, and degradation, can be disrupted under conditions such as oxidative stress (Korovila et al. 2017). Due to their direct exposure to the external environment, nasal epithelial cells are particularly vulnerable to oxidative stress (Yamamoto et al. 2023), which may lead to proteostatic imbalance. Recently, widespread amyloid protein aggregation was identified as a hallmark of aging across multiple human organs (Ding et al. 2025); however, whether similar protein aggregation occurs in the nasal epithelium remains to be determined. In support of this possibility, pathological accumulation of amyloid- β and PHF-tau has been reported in the olfactory epithelium of aged individuals, although the absence of tissue from younger donors in that study limits direct comparison (Arnold et al. 2010). Notably, hallmarks of aging in the nasal epithelium appeared distinct from those observed in the lung as, consistent with earlier reports (Minagawa et al. 2011), we did not detect a consistent age-related increase in SA- β -Gal staining in the epithelium of aging lungs.

Overall, our findings suggest that TIF are unlikely to serve as universal drivers of cellular senescence, which appears to arise through diverse mechanisms across different human cell types (Cohn et al. 2023). Future research involving human tissues, biopsies, or ex vivo cellular analyses will be essential to more accurately elucidate the mechanisms of cellular senescence in the human body, as traditional in vitro models of replicative senescence do not fully capture the complexity of senescence as it occurs in vivo.

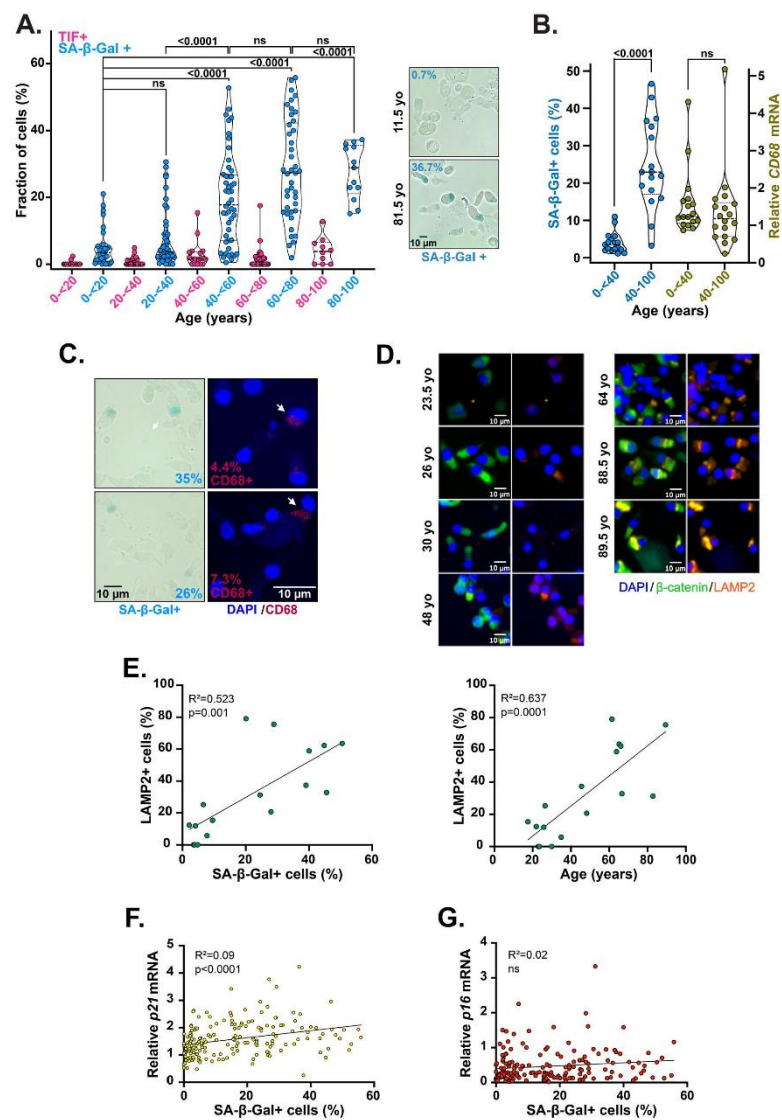


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FIGURE 4 | Early proteostasis disruption in nasal epithelial cells. (A) Percentage of SA- β -Gal-positive cells (SA- β -Gal+, blue) in nasal brushing samples and percentage of nuclei showing at least one TIF per nucleus (TIF+, pink) across the specified age ranges. For SA- β -Gal staining, on average, around 500 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal–Wallis tests. ns: Not significant. Right: Representative images of SA- β -Gal staining from two donors aged 11.5 and 81.5 years. Scale bar: 10 μ m. (B) Percentage of SA- β -Gal-positive cells (blue) and relative *CD68* mRNA expression (khaki) in a subset of nasal brushing samples from donors below or above 40 years old. *CD68* mRNA levels were normalized to *ACTB* mRNA and to a reference sample. Mann–Whitney tests. ns: Not significant. (C) Left: Representative images of SA- β -Gal staining in two nasal brushing samples with the indicated percentages of SA- β -Gal+ cells. Right: CD68 detection by immunofluorescence (red) on the same nasal brushing samples. DNA is stained with DAPI (blue). The percentage of CD68-positive cells (arrows) is indicated. Scale bar: 10 μ m. (D) Representative images of LAMP2 staining (orange) in nasal brushing samples from donors at the indicated ages. Cytoplasm is detected by β -catenin staining (green). DNA is stained with DAPI (blue). Scale bar: 10 μ m. (E) Linear regression analyses showing the percentage of LAMP2+ cells plotted against either SA- β -Gal+ cells (left) or donor age (right). (F, G) Linear regression analyses showing *p21* (F) or *p16* (G) expression levels in nasal brushing samples plotted against the percentage of SA- β -Gal+ cells. ns, not significant.

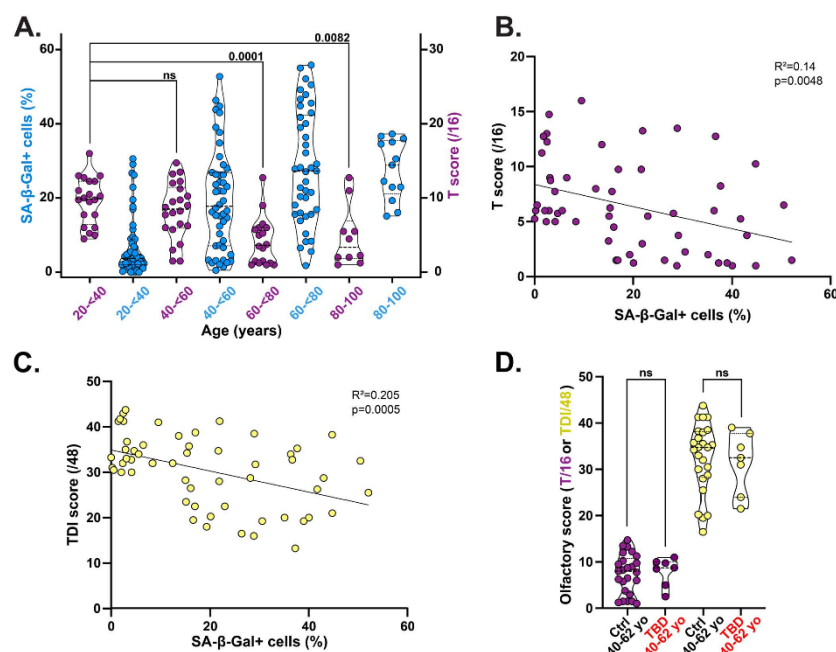


FIGURE 5 | SA- β -Gal activity in nasal epithelial cells negatively correlates with olfactory function. (A) Percentage of SA- β -Gal+ cells (blue, same as Figure 4A) in nasal brushing samples and Threshold (T) olfactory score (mauve) in donors across the specified age ranges. Violin plots showing median, first and third quartile. Kruskal–Wallis tests. ns, not significant. (B) Linear regression analysis of T score relative to the percentage of SA- β -Gal+ nasal brushing cells in a total of 75 donors aged 18–97 years. (C) As in panel B, but for the TDI (Threshold + Discrimination + Identification) olfactory score. (D) T (mauve) or TDI (yellow) olfactory scores in age-matched Ctrl and TBD patients. Violin plots showing median, first and third quartile. Unpaired *t* tests. ns, not significant.

Author Contributions

C.C., K.B., M.M., K.N., D.H., and Am.D. performed the experiments. S.E.V., L.D.S., A.V., W.W., J.V.S., and L.J.C. provided lung samples. D.M.B. supervised the STELA analyses. C.C. recruited the volunteers for nasal brushing with the help of An.D., A.F., and M.V.G. C.C. performed the nasal brushing and C.C. and K.B. performed the olfactory tests. C.H. contributed to nasal brushings and olfactory tests. C.C., K.B., and M.M. prepared the figures and participated in writing the manuscript. An.D. supervised the study and wrote the manuscript. All authors approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Senescence markers in aging human lungs. (A) Representative images of TIF analysis in the airway epithelium from Ctrl donors at the indicated age. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Scale bar: 10 μ m. (B) qRT-PCR analysis of *p16* expression in lung samples of donors across the specified age ranges. *p16* mRNA levels were normalized to *ACTB* mRNA and to one IPF lung sample used as control. Violin plots showing median, first and third quartile. Unpaired *t* test. ns: not significant. (C) Linear regression analysis of *p16* expression levels in lung samples relative to donor age. **Figure S2:** Hallmarks of cellular senescence in aged and IPF lungs. (A) Representative images of TIF analysis in the airway epithelium of IPF lungs from donors at the indicated age. Telomeres are detected with an antibody against TRF2 telomere-binding protein (red) and DNA damage is detected with an antibody against γ -H2AX (green). DNA is stained with DAPI (blue). Scale bar: 10 μ m. (B) Representative images of TIF analysis in the airway and alveolar epithelium of lung samples from a 75-year-old control donor and a 60-year-old IPF donor. Telomeres are detected with a telomeric FISH probe (red), DNA damage is detected with an antibody against γ -H2AX (green) and AT2 cells are detected with an antibody against pro-SPC (white). DNA is stained with DAPI (blue). Scale bars: 10 and 5 μ m. (C) Mean frequency of AT2 and non-AT2 cells in the alveolar epithelium of aging lungs (75–83 years old, *n* = 4) and IPF lungs (60–61 years old, *n* = 4) containing at least one TIF. On average, approximately 60 AT2 nuclei and 140 non-AT2 nuclei were analyzed per sample in the alveolar epithelium. For the airway epithelium of IPF lungs, the mean frequency of TIF-positive cells was determined from approximately 50 nuclei per sample. Violin plots showing median, first and third quartile. One-way ANOVA. ns: not significant. (D) Representative images of p16 staining by immunohistochemistry in IPF lung samples from two 60-year-old donors. HE: Hematoxylin-Eosin. Scale bar: 50 μ m. **Figure S3:** Age distribution of control donors providing nasal brushing samples. **Figure S4:** No significant increase in total γ -H2AX signals in aging human nasal epithelial cells. Mean number of total γ -H2AX foci per nucleus in the nasal brushing samples of donors across the specified age ranges. On average, around 65 nuclei were analyzed per sample. Violin plots showing median, first and third quartile. Kruskal-Wallis test. ns: not significant. **Table S1:** Lung samples used in this study. **Table S2:** Volunteers for nasal brushing and/or olfactory tests. **Table S3:** Antibodies used in this study. All antibodies were used in IF experiments. **Table S4:** Primers used in this study for qRT-PCR. All primers were obtained from Eurogentec (Belgium).