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Virtual histology of Alzheimer's Disease: Biometal entrapment within amyloid- β plaques allows for detection via X-ray phase-contrast imaging

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Abstract

Amyloid- β (A β) plaques from Alzheimer's Disease (AD) can be visualized *ex vivo* in label-free brain samples using synchrotron X-ray phase-contrast tomography (XPCT). However, for XPCT to be useful as a screening method for amyloid pathology, it is essential to understand which factors drive the detection of A β plaques. The current study was designed to test the hypothesis that A β -related contrast in XPCT could be caused by A β fibrils and/or by metals trapped in the plaques. Fibrillar and elemental compositions of A β

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plaques were probed in brain samples from different types of AD patients and AD models to establish a relationship between XPCT contrast and A β plaque characteristics. XPCT, micro-Fourier-Transform Infrared spectroscopy and micro-X-Ray Fluorescence spectroscopy were conducted on human samples (one genetic and one sporadic case) and on four transgenic rodent strains (mouse: APPPS1, ArcA β , J20; rat: TgF344). A β plaques from the genetic AD patient were visible using XPCT, and had higher β -sheet content and higher metal levels than the sporadic AD patient, which remained undetected by XPCT. A β plaques in J20 mice and TgF344 rats appeared hyperdense on XPCT images, while they were hypodense with a hyperdense core in the case of APPPS1 and ArcA β mice. In all four transgenic strains, β -sheet content was similar, while metal levels were highly variable: J20 (zinc and iron) and TgF344 (copper) strains showed greater metal accumulation than APPPS1 and ArcA β mice. Hence, a hyperdense contrast formation of A β plaques in XPCT images was associated with biometal entrapment within plaques.

Keywords: Neurodegenerative diseases, Alzheimer’s disease, Amyloid- β plaques, X-ray phase-contrast tomography, Fourier-Transform Infrared spectroscopy, X-Ray Fluorescence spectroscopy

1. Introduction

Amyloid- β (A β) plaques are an early hallmark of Alzheimer’s Disease (AD) in the human brain. A β plaques are formed by the aggregation of amyloid- β peptides with altered conformation into fibrils. Despite advances provided by in vivo imaging techniques such as A β positron emission tomography (PET) [1], AD diagnosis still requires an autopsy to be confirmed. The *postmortem* examination of brain tissue results in a neuropathological scoring, like the NIA-AA “ABC” score [2] — which pools results from CERAD A β score (A β density) [3], Braak’s stages (tau spreading) [4] and Thal-Braak phases (A β spreading) [5] into a final AD probability. X-ray phase-contrast tomography (XPCT) refers to a family of techniques that use the phase shift rather than the attenuation of X-rays to probe the microstructure in soft biological tissues. X-ray 3D imaging of A β plaques has been described in transgenic rodent models that develop amyloidosis for the past decade, using propagation-based phase contrast (either with synchrotron radiation [6–11] or with a laboratory source [12]) or using grating-based phase contrast (with synchrotron radiation [6, 13–16]). These techniques generate 3D images with

an isotropic resolution ranging from 10 nm [17] to 10 μm , thus providing a so-called “virtual histology” of the excised brain [18–20] with minimal preparation (fixation, dehydration or paraffin embedding). Imaging AD samples with XPCT is particularly interesting because plaques stand out in the images without the need to add any staining agents. Previous studies in transgenic animals highlighted the great potential of XPCT for the quantitative analysis of amyloid- β plaques (overall burden [6, 15], spatial distribution [8], 3D morphometry [10]). These advances (which bring original information not available to standard immunohistochemistry), combined with increasingly faster acquisitions (less than 5 min per sample [10]), position XPCT as a potential screening tool for amyloid pathology. Overall, the ability to perform a virtual histology of A β pathology with XPCT has been reported in 13 papers. But none of these papers studied the source of the contrast. Hence, up to now, the substrate underlying contrast of A β plaques in XPCT images remains unexplored. In other words, it is not known why some A β plaques, especially in transgenic AD models, are detected with XPCT.

Phase-contrast signal emerges from local changes of the X-ray refractive index within the plaques — compared to that of the surrounding tissue —, which makes them stand out. Changes of the refractive index could be caused by the characteristics of the fibrils structured as β -sheet stacks of amyloid- β peptides [21–23], which form dense and relatively insoluble aggregates. Or they could be linked to the entrapment of endogenous metals within the plaques, as iron, copper and zinc have been observed as co-localized with A β plaques, both in the human pathology [21, 24] and in rodent models [25–27]. We tested these two non-exclusive hypotheses to elucidate the origin of the phase-contrast signal of A β plaques. To this end, we performed an examination of the same excised brains from a variety of transgenic rodents (four transgenic strains) and AD patients (one sporadic case and one familial case) with three different and complementary synchrotron techniques, namely:

- i*) XPCT to visualize the plaques and measure the contrast with surrounding tissue,
- ii*) Fourier-transform infrared microspectroscopy (μFTIR) to quantify the proportion of β sheets in the plaques,
- iii*) X-ray fluorescence microspectroscopy (μXRF) to assess metal quantities in the plaques.

We here report new XPCT-assessed densities of A β plaques: in rodents, some strains were hypodense while others had the already described hyperdensities. In humans, XPCT highlighted parenchymal A β plaques for the first time (in the genetic patient). Overall our results suggest that XPCT hyperdensities are associated with the levels and spatial distributions of metal entrapment.

2. Material & Methods

2.1. Study design

Figure 1 summarizes study design, which intended to examine A β plaques from the same brains with XPCT, μ FTIR and μ XRF. Tissue preparation was not compatible between the three techniques (μ XRF being considered sensitive to fixation [28–30]), thus the samples had to be cut: one half was processed for XPCT, the other half was processed for μ FTIR followed by μ XRF. The list of samples, and all measurements — included and discarded (outliers for μ XRF or Mie artifacts for μ FTIR) — performed on individual A β plaques ($n = 9$ –23 per strain), is available as a supplementary file.

2.2. Samples

2.2.1. Human brain samples

Human brain samples (from parietal and frontal cortex) and associated data were obtained from Tissu-Tumorotheque Est Biobank (CRB-HCL Hospices Civils de Lyon) authorized by the French Ministry of Research (authorizations DC2008-72 & AC2015-2576):

- A 51-year-old male patient with genetic (APP duplication) AD (post-mortem delay 36 h);
- An 82-year-old female patient with sporadic AD (post-mortem delay 11 h).

Both had pronounced A β pathology (Thal–Braak stage V, CERAD score 3) [31], and had been previously diagnosed as free from prion disease.

Two snap-frozen samples were collected. One sample was kept frozen for sectioning, while the other was fixed with formaldehyde 4% (as described in the tissue preparation section below).

Contralateral formalin-fixed paraffin-embedded (FFPE) blocks were cut onto slides and stained with multiple agents:

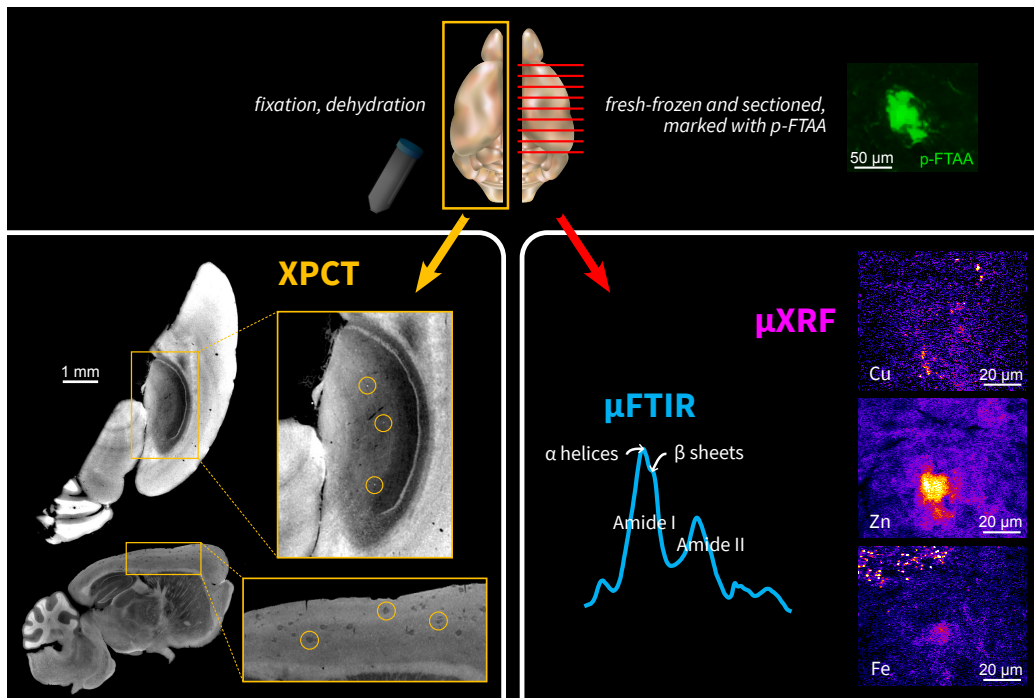


Figure 1: Experimental design, summarizing sample preparation and imaging techniques. A half of the sample was fixed and dehydrated then scanned in 3D with XPCT (left part). The other half was sectioned while frozen, fluorescently stained to locate A β plaques, and analyzed in 2D using μ FTIR (for β -sheet load) and μ XRF (for metal level).

- Anti-A β A4 antibodies for A β load;
- Anti-phosphorylated-tau antibodies for neurofibrillary tangles (NFTs) and p-tau-positive neuritic plaques;
- Luxol fast blue and periodic acid-Schiff (PAS) stains for myelin and glycogen-rich bodies.

2.2.2. Transgenic rodent brain samples

Four transgenic rodent models that develop amyloidosis were studied:

- Mono-transgene line ArcA β ($n = 3$ mice, 15 months old), with mutant APP [32]

(<https://www.alzforum.org/research-models/arcav>)

- Double-transgene line APPPS1 ($n = 3$ mice, 12 months old), with mutant APP and PSEN1 [33]
(<https://www.alzforum.org/research-models/appps1>)
- Mono-transgene line J20 ($n = 2$ mice, 13 months old), with mutant APP [34]
(<https://www.alzforum.org/research-models/j20-pdgf-appswind>)
- Double-transgene line TgF344 ($n = 3$ rats, 20 months old), with mutant APP and PSEN1 [35]
(<https://www.alzforum.org/research-models/tgf344-ad>)

One wild-type (WT) rat (Fischer 344) and one WT mouse (C57Bl/6), respectively 20 and 15 months old, were used as controls.

Rodents were sacrificed with intracardiac PBS perfusion to clean blood vessels. Brains were excised and kept at -80°C .

Mouse brains were cut in half along the hemisphere (Figure 1). One half was then fixed and dehydrated (for XPCT). The other half was immediately put back at -80°C (for μFTIR and μXRF).

Only one hemisphere was available per rat brain. The hemispheres were cut in the middle of the hippocampus. One half was then fixed and dehydrated (for XPCT). The other half was immediately put back at -80°C (for μFTIR and μXRF).

2.2.3. Fixed tissue and dehydration

The unfrozen samples were fixed for 24 to 48 hours with 4% FA. Then they were dehydrated at RT in a series of 8 baths with a gradient of ethanol concentrations, diluted with PBS, for 5 minutes each: 25%, 25%, 50%, 50%, 75%, 75%, 96%, 96%. Finally, they were put into plastic tubes filled with 96% ethanol and kept at 4°C .

2.2.4. Frozen tissue and preparation of SiRN membranes

The frozen samples were dissected into blocks in the hippocampus / upper cortex area, to ensure they would fit into an area of $4 \times 4 \text{ mm}^2$. Then they were sectioned using a Leica CM1850 cryostat at -20°C with a thickness of $7 \mu\text{m}$. PTFE-coated blades (DuraEdge™, TedPella, Inc., USA) were used to prevent metallic contamination. Brain sections were placed with a paintbrush on 200 nm-thick silicon-rich nitride (SiRN) membranes (Silson Ltd, UK) with

an inner frame side of 4 millimeters. Sections were stained for 5 minutes with a pan-amyloid fluorescent agent, p-FTAA [36] ($1 \mu\text{mol L}^{-1}$ in PBS with 0.01 % NaN_3); membranes were then rinsed once with nanopure water. Fluorescence and bright-field transmission images were acquired with a ZEISS microscope (cf. Figure 2A). Afterwards, membranes were kept in a desiccator with silica gel blue (2 mm–4 mm, Roth) until synchrotron experiments (1–4 weeks after).

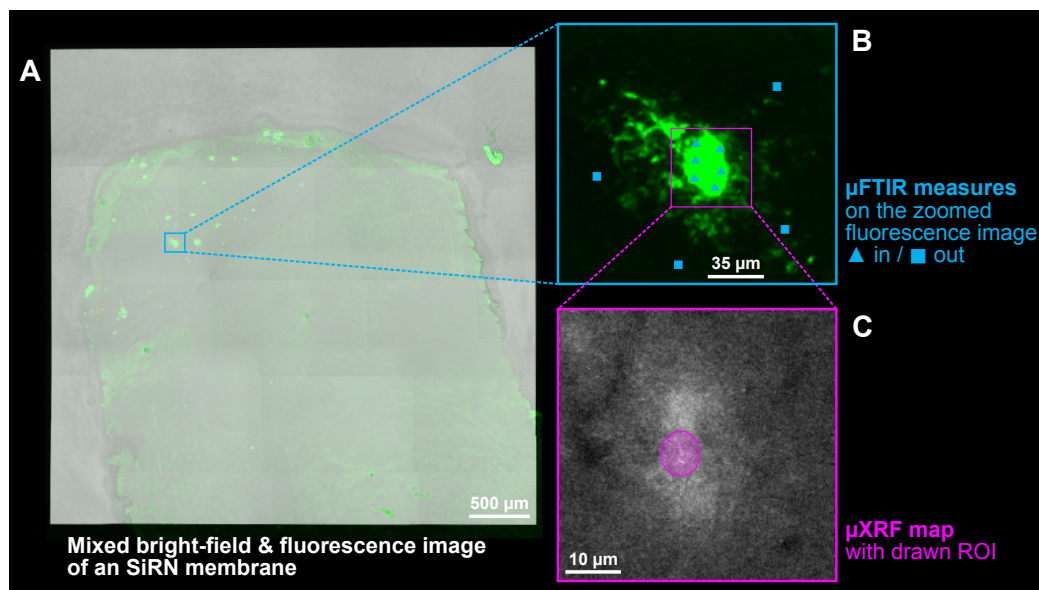


Figure 2: Silicon-nitride membrane overview and 2D imaging with μFTIR and μXRF . (A) Sectioned samples were placed on SiRN membranes, fluorescently stained and imaged with a microscope with 2 channels: bright-field and green fluorescence. (B) During the experiment at the SMIS beamline, the fluorescence was used to target $\text{A}\beta$ plaques and multiple μFTIR spectra were acquired within individual plaques and in the surrounding tissue. (C) During the experiment at the NANOSCOPIUM beamline, μXRF hypermaps were acquired on the same $\text{A}\beta$ plaques — here shown as the sum over the whole spectrum.

2.3. Synchrotron experiments

All experiments, unless explicitly stated, were conducted at the synchrotron radiation facility SOLEIL, within the scope of the proposal #20200258.

2.3.1. X-ray phase-contrast tomography (XPCT)

Formaldehyde-fixed, ethanol-dehydrated samples were imaged with X-ray phase-contrast tomography (XPCT) at the ANATOMIX beamline [37] with

a pink beam of mean energy 22 keV and a detector pixel size of 6.5 μm . The detector system consisted of a lutetium aluminum garnet (LuAG, $\text{Lu}_3\text{Al}_5\text{O}_{12}$) single-crystal scintillator (600 μm thick) coupled to a CMOS camera (Hamamatsu Orca Flash 4.0 V2, 2048×2048 pixels) via a 1 : 1 lens system consisting of two Hasselblad photo objectives ($f = 100$ mm) in tandem geometry. Complete acquisition parameters are reported in Supplemental Table 1.

For reconstruction using PyHST2 [38], we set the Paganin length parameter to 552 μm ($\delta/\beta = 1525$). We used a motion blur correction algorithm and an unsharp mask filter ($c_{unsharp} = 0.5$, $\sigma_{unsharp} = 2$ px).

Human samples were imaged with a smaller pixel size (3.1 vs. 6.5 μm) via a 1.45 : 1.45 lens system using a similar setup, during a second session. The Paganin length was adapted to 216.65 μm ($\delta/\beta = 1400$).

2.3.2. XPCT image analysis

Ring artifacts were removed using an in-house tool implemented by the company NOVITOM (France; <https://www.novitom.com/en/>) [20]. Samples were isolated from the background (air, plastic tube and ethanol) and rotated within the AMIRA 3D software (release 2021.1, Thermo Fisher Scientific, USA). A β plaques were segmented semi-automatically using a random forest algorithm from the IPSDK software (Reactiv'IP, France; <https://www.reactivip.com/>). The generated masks were screened for false positive segmentations and ~ 20 plaques were selected, along with ROIs of the surrounding tissue as reference. Signal intensity measurements — as a ratio of the mean intensities of the plaques and the mean intensities of the reference ROIs — and volumes were retrieved with MorphoLibJ [39], an ImageJ plugin.

2.3.3. Fourier-transform infra-red microspectroscopy (μFTIR)

The brain cryosections on the membranes (cf. Figure 2B) were analyzed by synchrotron-radiation FTIR microspectroscopy with a Continuum XL microscope and a Thermo Nicolet 6700 bench at the SMIS beamline. The microscope was equipped with a fluorescence imaging setup and an halogen mercury lamp for excitation of the p-FTAA fluorescent probe that binds A β plaques.

Spectra on and around the spots of interest (respectively referred as “In” and “Out”) were recorded between 650 cm^{-1} and 4000 cm^{-1} at 4 cm^{-1} in $10 \times 10 \mu\text{m}^2$ dual-aperture mode, using a Schwarzschild 32 \times (NA 0.65) objective and a matching condenser; zero-padding enhanced resolution to

0.5 cm^{-1} . All parameters are shown in Supplemental Table 2. Spectra were pre-processed with the atmospheric suppression algorithm from OMNIC 9.2 (Thermo Fisher Scientific, USA) to remove water vapor contribution.

Besides, a few acquisitions were performed as evenly-spaced arrays (“maps”) with a lower co-addition to scan larger areas on a few samples, especially those devoid of $A\beta$ from the wild-type animals.

2.3.4. μ FTIR spectral analysis

Spectra were processed using Quasar [40]. All the data were imported and processed with the same pipeline:

Cutting: The region from 650 cm^{-1} to 800 cm^{-1} was cut out because of high-frequency noise;

Quality assessment: Spectra were thoroughly screened for Mie scattering artifacts — indeed, Mie scattering is responsible for the shifting of the Amide I peak towards lower wave numbers and for its distortion, which leads to measurements that are easily mistaken for higher β -sheet content [41];

Absorbance thresholding: MCT detectors have a limited range for linearity between 0.2 and 1.4, so spectra with a peak-to-peak amplitude outside this range were discarded;

Baseline correction: A linear baseline correction was applied on the Amide I band, from 1415 cm^{-1} to 1770 cm^{-1} ;

Feature selection: Absorbance values closest to 1655 cm^{-1} (non- β sheets) and 1630 cm^{-1} (β sheets) [42] were used as features to compute a spectroscopic ratio for amyloid β -sheet content: $\frac{A_{1630 \text{ cm}^{-1}}}{A_{1655 \text{ cm}^{-1}}}$

2.3.5. X-ray fluorescence microscopy (μ XRF)

The brain cryosections on the membranes (cf. Figure 2C) were analyzed by x-ray fluorescence (μ XRF) microscopy on the “CX3” custom bench at the NANOSCOPIUM beamline, using the “flyscan” mode with steps of $0.3 \mu\text{m}$. Measures were performed on the same locations on the membranes as for μ FTIR acquisitions (cf. § 2.3.3 above). The matching locations were obtained according to the Supplemental Methods. The inline optical bright-field microscope which is mounted on the beamline helped fine-tuning the position. All parameters are shown in Supplemental Table 3.

Three standard reference materials (SRMs) were also scanned: the SRM 1577c “bovine liver” and SRM 1832 (NIST, USA), along with the “RF” standard thin film (AXO DRESDEN GmbH, Germany).

2.3.6. μ XRF elemental analysis

Spectra were processed using PyMca [43]. First, the raw spectral maps were summed to locate the plaques more precisely within the scanned field of view — from $20 \times 20 \mu\text{m}^2$ to $80 \times 80 \mu\text{m}^2$. Regions of Interest (ROIs) were drawn at the location of each plaque.

Then, the measures on the three SRMs and on a blank SiRN membrane helped the calibration of the software — using the fundamental parameter method — and the batch-fitting tool computed the mass fraction maps for each element. Reported mass fractions in $\mu\text{g g}^{-1}$ or “parts per million” (ppm) were obtained by averaging within the drawn ROI. One can easily retrieve an approximate surface concentration: $10 \text{ ppm} \approx 1 \text{ ng cm}^{-2}$; because $e_{\text{dried tissue}} \approx 1 \mu\text{m}$ and $\rho_{\text{dried tissue}} = 1.04 \text{ g cm}^{-3}$ (the density of dried brain tissue).

2.4. Statistics

The statistical analysis was performed with GraphPad Prism version 8.4.3 for Windows (GraphPad Software, California USA; www.graphpad.com). Results are displayed as box plots which use the Tukey standards, i.e. the whiskers end at $Q1 - 1.5 \times IQR$ and $Q3 + 1.5 \times IQR$ (IQR is the interquartile range); values outside of $[Q1 - 1.5 \times IQR; Q3 + 1.5 \times IQR]$ are represented as points; means are represented as crosses and medians as a wide line. Reported fold increases are computed between the means.

The normality and log-normality D’Agostino-Pearson tests failed for the whole dataset. Kruskal-Wallis tests with post-hoc Dunn’s multiple comparison tests were performed on XPCT intensities, μ FTIR-derived data (without wild-type data because of the different acquisition process) and μ XRF-derived data in rodents. A separate Kruskal-Wallis test with post-hoc Dunn’s multiple comparison tests was performed for human μ FTIR-derived data. For human μ XRF-derived data, a non-parametric Mann-Whitney test was performed per element (iron, zinc and copper levels were considered independent).

3. Results

3.1. Genetic and sporadic human AD samples exhibit different A β contrasts in XPCT

On XPCT images (Figure 3), the multiple A β plaques from the genetic AD patient were clearly visible in grey matter, exhibiting a typical neuritic aspect (Figure 3, A1, yellow arrow heads) and diameter around 50 μm . Anti-A β staining of slices derived from contralateral FFPE block confirmed the presence of numerous plaques with dense central core and less compact peripheral halo (Figure 3, B1, yellow arrows). The central core of neuritic plaques and surrounding dystrophic neurites containing neurofibrillary material were also stained using anti-p-tau antibodies (Figure 3, C1, yellow arrows). The presence of multiple A β plaques in this familial form of AD was especially striking when scrolling through the virtual sample (see Supplemental Movies). In contrast, the numerous A β diffuse plaques detected in A β -stained slides from the sporadic AD patient could not be reliably visualized in XPCT (Figure 3, red arrow heads). In both genetic and sporadic AD cases, smaller, round-shaped and highly hyperdense structures were observed in sub-pial layer of frontal sulcus. These structures were presumably corporea amylacea, as far as similarly shaped inclusions positive to periodic acid-Schiff (PAS) staining were found in similar location in slides from FFPE contralateral blocks for both genetic and sporadic AD (Figure 3, blue arrows). Other variably hyperdense structures were neuronal bodies and vessel walls.

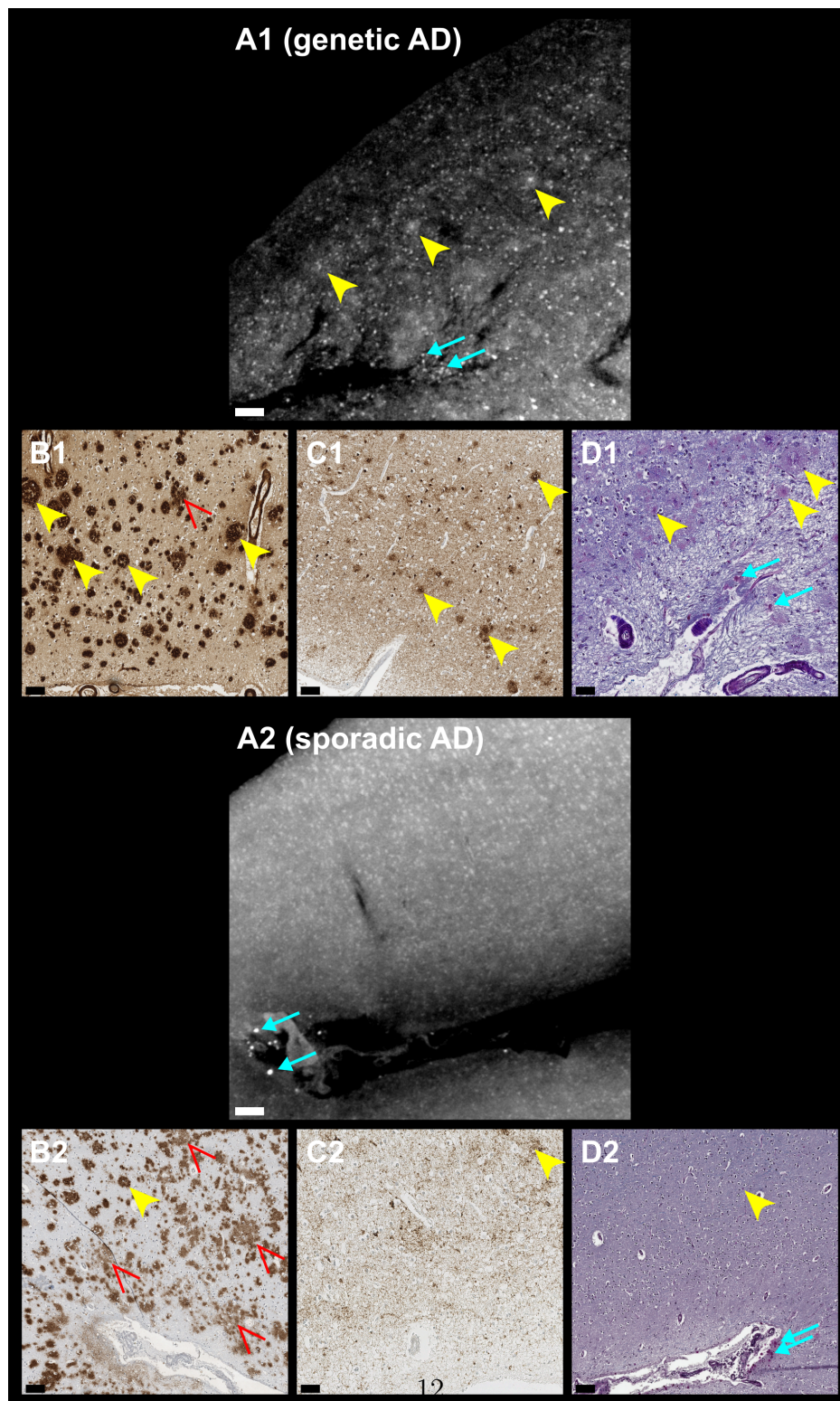


Figure 3: (caption on next page)

Figure 3: Representative XPCT images of brain tissue with A β plaques from a genetic (A1) and a sporadic (A2) cases of Alzheimer’s Disease. A1&A2 are maximum intensity projections (MIPs) over 5 slices (16 μ m); for raw images, please refer to Supplemental Movies. Virtual slices are compared to contralateral FFPE slices (genetic AD case: B1–D1; sporadic AD case: B2–D2): anti-A β A4 immunostaining (B1&B2), anti-p-tau immunostaining (C1&C2) and LFB / PAS staining (D1&D2). Yellow arrow heads point at neuritic amyloid plaques while red arrow heads point at diffuse plaques. Other hyperdensities, smaller than A β plaques, were corporea amylyacea (blue arrows). Neuron soma and blood vessel walls were slightly hyperdense. Scale bars equal 100 μ m.

3.2. β -sheet content and metal entrapment are strikingly enhanced in the genetic case compared to the sporadic case

As expected, the β -sheet content in the fluorescence-labelled plaques (Figure 4A) was higher than in the surrounding tissue for both patients (genetic AD: +50 %; sporadic AD: +4 %). Besides, the β -sheet content significantly differentiated A β plaques between the genetic and sporadic AD patients (genetic AD: +55 %; $p = 0.03$).

Levels of metals within A β plaques (Figure 4B) were higher (iron: +109 %; zinc: +97 %; copper: +88 %) for the genetic AD patient compared to the sporadic AD patient.

3.3. Transgenic AD models exhibit a variety of A β contrasts in XPCT

To further investigate the relationship between XPCT A β contrast and A β composition, we used 4 different transgenic rodent strains (Figure 5): the J20 mice and the TgF344 rats had small hyperdense plaques (typically in the range \varnothing 25–30 μ m for J20; and \varnothing 15–20 μ m for TgF344) mostly seen in the hippocampus (see Supplemental Movies), while the APPPS1 and ArcA β strains had numerous and large hypodense plaques (APPPS1: \varnothing 40–45 μ m; ArcA β : \varnothing 45–60 μ m), which however frequently exhibited a hyperdense core (APPPS1: \varnothing 10–15 μ m; ArcA β : \varnothing 15–20 μ m), and were mainly located in the cortex but also in the hippocampus. Signal intensity measurements from \sim 20 segmented plaques per sample are shown in Figure 5B.

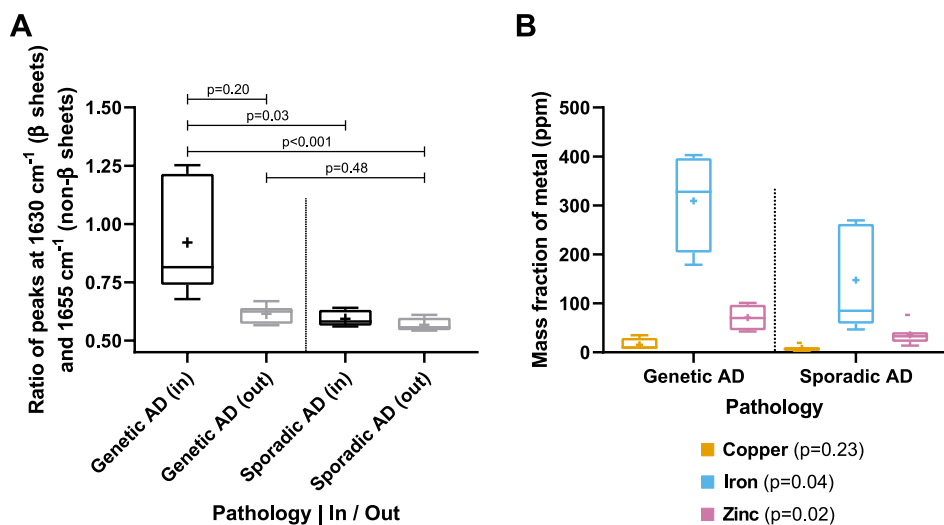


Figure 4: (A) Amyloid β -sheet content and (B) metal levels within $A\beta$ plaques from Alzheimer's Disease patients. β -sheet content is computed from peak ratios on μ FTIR spectra and averaged per plaque ($n = 6$ for each patient). Metal levels are obtained by peak fitting on μ XRF hyperspectra and averaged within a ROI on a plaque ($n = 4$ for the genetic AD, $n = 7$ for the sporadic AD). A Kruskal-Wallis test ($p = 0.0011$) with post-hoc Dunn's multiple comparison tests (p displayed on graph) were performed for μ FTIR data and a per-metal non-parametric Mann-Whitney test (p displayed in legends) was performed for μ XRF (iron, zinc and copper levels were considered independent).

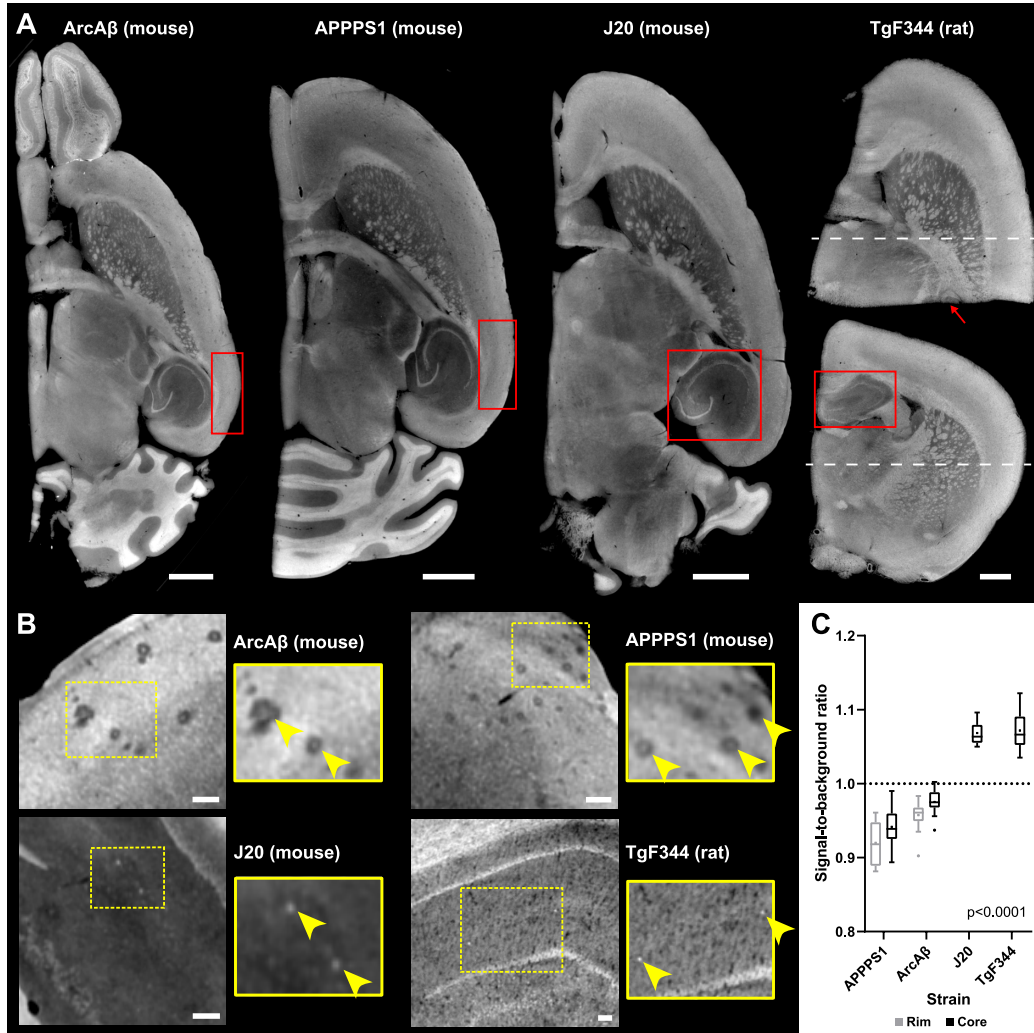


Figure 5: (A) XPCT single-slice axial views of whole samples. Red rectangles and arrow show the analyzed areas for each strain. For the rat sample, the hemisphere was cut in half and the dotted line in the (upper) axial view shows the section plane for the (lower) coronal view. (B) XPCT slices of brain tissue with A β plaques from transgenic rodents and (C) associated local contrasts of A β plaques. Insets are zoomed single-slice ROIs. Plaques from ArcA β and APPPS1 mice are dark (hypodense) and mainly observed in the cortex. Plaques from J20 mice and TgF344 rats are bright (hyperdense) and mostly located in the hippocampus. Kruskal-Wallis tests of signal-to-background ratio between bright-plaque strains and dark-plaque strains yielded significant differences ($p < 0.0001$). Scale bars equal 1 mm in (A) and 100 μ m in (B).

3.4. β -sheet content is constant across transgenic strains while metal entrapment is highly variable

The contralateral hemispheres were used to study fibrillar and elemental composition of these plaques. The β -sheet content (Figure 6A) increased in fluorescently-labelled spots in the rodent brain slices (measurements “In” vs. “Out” of fluorescent A β plaques: +37 % for APPPS1, +36 % for ArcA β , +38 % for J20, +47 % for TgF344). However, there were no difference of fibrillar content (“In”) between the four strains of animals. The β -sheet content in the surrounding tissue (“Out”) in transgenic animals was similar to the level in corresponding wild-type animals (APPPS1: +6 %; ArcA β : +2 %; J20: +9 %; TgF344: -6 %).

All transgenic strains also had increased levels of iron, zinc and copper at the analyzed A β plaques compared to their wild-type counterparts. However, μ XRF-measured metal composition of the A β plaques (Figure 6B) varied between the different strains. J20 mice had the highest levels of zinc and iron compared to the other strains (zinc: +583 % vs. WT; iron: +803 % vs. WT) and TgF344 rats had the highest level of copper (+770 % vs. WT). Meanwhile, APPPS1 mice had the lowest mean levels and ArcA β mice had intermediate levels.

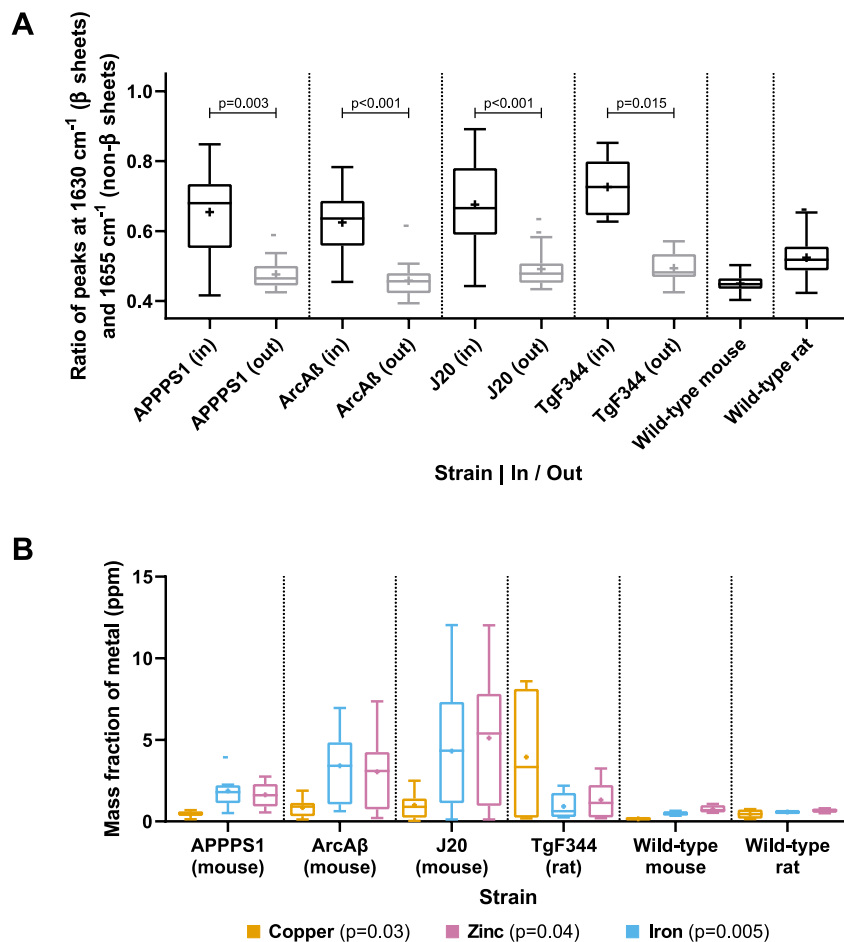


Figure 6: (A) Amyloid β -sheet content and (B) metal levels within $A\beta$ plaques and in the surrounding tissue from transgenic rodents. β -sheet content is computed from peak ratios on μ FTIR spectra and averaged per plaque ($n = 9$ – 20 plaques per strain). Metal levels are obtained by peak fitting on μ XRF hyperspectra and averaged within a ROI on a plaque ($n = 4$ – 17 plaques per strain). For μ FTIR, a Kruskal-Wallis test ($p < 0.0001$) with post-hoc Dunn's multiple comparison tests (p displayed on graph) was performed between transgenic strains (i.e. without wild-type mouse and wild-type rat); for μ XRF, a per-metal Kruskal-Wallis test (p displayed in legends) was performed (iron, zinc and copper levels were considered independent).

3.5. Comparison between human and rodent datasets

Figure 7 shows a qualitative comparison of the multimodal dataset obtained with XPCT, μ FTIR and μ XRF, between transgenic rodents and humans. XPCT (left row) highlighted 3 distinct types of A β plaques: small hyperdense plaques in J20 mice and TgF344; larger, hypodense plaques with a dense core in ArcA β and APPPS1; and similarly large, but diffusely hyperdense plaques in the genetic case of AD. All types of plaques exhibited an increased level of β -sheet content which matched the fluorescence signal, as measured by μ FTIR (central row), and which tended to be increased in the human case compared to animal models. The accumulation of endogenous metals (right row) appeared mainly restricted to the (hyperdense) core of the plaque in rodents, while it appeared consistently diffuse over the whole μ XRF FOV in the human plaques.

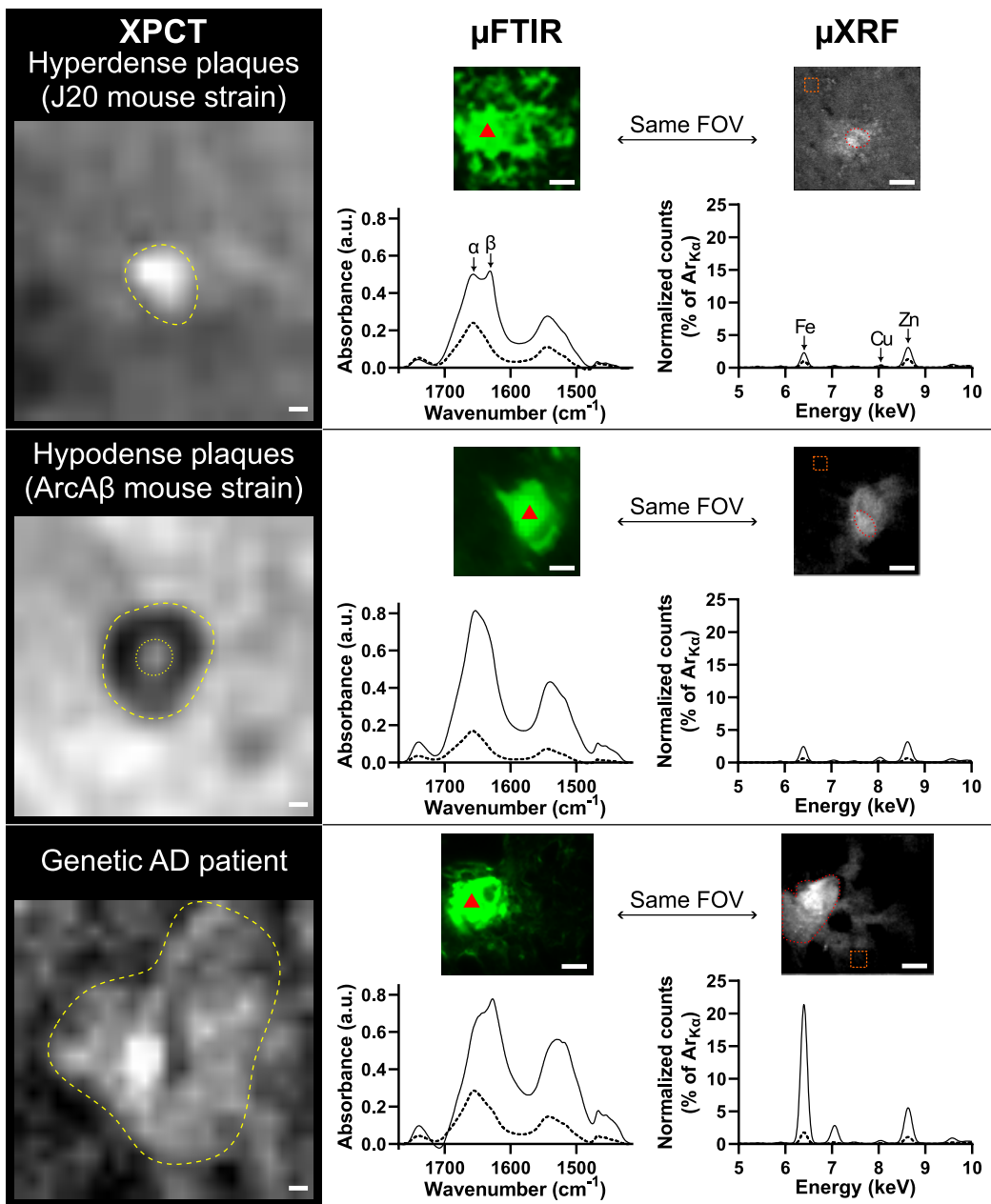


Figure 7: (caption on next page)

Figure 7: Multimodal dataset obtained with the three techniques on A β plaques. XPCT scans (left column) were acquired on an unsectioned sample, while FTIR (center) & XRF (right) hyperspectra were both acquired on a separate sectioned sample. Inline bright-field microscopy was used to match the fields of view (FOVs) between μ FTIR and μ XRF. Representative measurements in the plaques are indicated with a red arrowhead (μ FTIR) and red outline (μ XRF), and plotted as plain lines. Measurements outside the plaques (μ FTIR: acquired in fluorescence-free regions outside the shown FOVs; μ XRF: orange outline) are plotted as dotted lines. In the case of μ FTIR, a linear baseline correction was applied on the Amide I band, from 1415 cm^{-1} to 1770 cm^{-1} . In the case of μ XRF, maps show the summed μ XRF signal; spectra were normalized with the K α peak of argon (Ar) and unlabelled peaks at $\sim 7.1\text{ keV}$ and $\sim 9.6\text{ keV}$ correspond to non-K α peaks of Fe and Zn, respectively; single-element maps for iron, copper and zinc and per-element fitting curves are shown in Supplemental Figure 1. Scale bars equal $10\text{ }\mu\text{m}$.

To verify this, the volumes of whole A β plaques and cores, segmented on XPCT, were compared to the areas of μ XRF used to extract metal measurements, and areas of fluorescent spots used for μ FTIR measurements. In Figure 8, areas of metal accumulation (derived from μ XRF) were similar across all 4 strains (shaded area, panel A). In contrast, whole-plaques volumes (derived from XPCT) clearly discriminated between the larger plaques of APPPS1 and ArcA β on one side and the smaller plaques of J20 and TgF344 on the other side. However, when focusing on hyperdensities, the hyperdense cores of APPPS1 and ArcA β (bright cores with dark corona) had similar XPCT volumes as the hyperdense plaques of J20 and TgF344 (bright cores without any corona). μ XRF-derived areas of metal accumulation are therefore in agreement with a hyperdense signal in XPCT.

In the human case with inherited AD, the A β plaques had a unique pattern showing both a hyperdense core with a hyperdense, diffuse corona. First, this was in line with μ XRF maps showing larger and more diffuse metal accumulation ($1020\text{ }\mu\text{m}^2 \pm 530\text{ }\mu\text{m}^2$), as compared with rodents ($43\text{ }\mu\text{m}^2 \pm 32\text{ }\mu\text{m}^2$, pooled measurements of all four strains). Second, fluorescent areas had a similar size as μ XRF ones for this genetic AD case ($1230\text{ }\mu\text{m}^2 \pm 780\text{ }\mu\text{m}^2$ vs. $1020\text{ }\mu\text{m}^2 \pm 530\text{ }\mu\text{m}^2$), but were much larger than μ XRF ones in rodents ($1260\text{ }\mu\text{m}^2 \pm 1010\text{ }\mu\text{m}^2$ vs. $43\text{ }\mu\text{m}^2 \pm 32\text{ }\mu\text{m}^2$, shaded area, panel B), an indication that fibrillation, in contrast to metal accumulation, was not systematically associated with a hyperdense signal in XPCT.

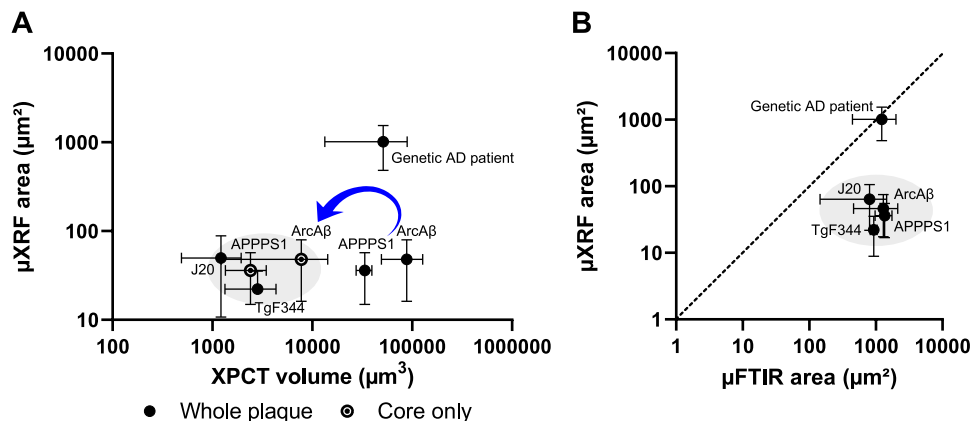


Figure 8: (A) Areas of μXRF ROIs for elemental mapping within the $\text{A}\beta$ plaques (sectioned sample) vs. volumes of segmented $\text{A}\beta$ plaques (contralateral unsectioned sample). Considering the cores alone in XPCT images yielded a higher correlation among the rodent data (blue arrow). (B) Areas of μXRF ROIs for elemental mapping vs. areas of fluorescence for μFTIR .

4. Discussion

The present study sought to provide a rationale for the detection for $\text{A}\beta$ plaques with synchrotron X-ray phase-contrast tomography, through a correlative approach between the local contrast — or signal intensity — and the fibrillar (μFTIR) and elemental (μXRF) compositions. The experimental design relied on three imaging modalities, all available at synchrotron SOLEIL, and included brain samples from patients diagnosed with Alzheimer’s Disease (AD) and transgenic rodents which developed amyloidosis. 2D correlative examination of $\text{A}\beta$ plaques through μFTIR and μXRF has been an active field of research for 15 years (e.g. [21, 25, 44, 45]), which constantly highlighted that local elevations of β -sheet content and metal trapping were concomitant features across a variety of AD specimens, including in cellular models [46]. However this is the first time that the composition of $\text{A}\beta$ plaques is linked with a 3D imaging method such as XPCT.

Using propagation-based imaging, now the most widely employed XPCT method for virtual histology of label-free samples, we made original observations. In animals, $\text{A}\beta$ plaques from the J20 mouse strain and the TgF344 rat strain appeared as small bright (hyperdense) spots, as already reported for these and for other transgenic models such as 3xTg [10], APP/PS1 [8, 10],

TauPS2APP [6] and B6C3-Tg [14]. But surprisingly, A β plaques in the newly imaged APPPS1 and ArcA β mouse strains appeared as dark (hypodense) spots, with the largest plaques frequently exhibiting a brighter (hyperdense) core. In humans, we confirmed previous observations that parenchymal A β plaques are usually not detected with XPCT, except rare deposits which were reported as calcified [19, 47]. However, we unveiled that A β densities in the human samples are more pronounced in an inherited case of AD — of which the first XPCT images are reported here — compared to sporadic AD. This variable appearance of A β aggregates is reminiscent of A β polymorphism, which describes, both in animal models and in humans, the existence of subtypes of A β plaques [48, 49].

FTIR microspectroscopy can be used to quantify the β -sheet content in individual A β plaques with exquisite sensitivity. Indeed, the ratio of the β -sheet over non- β -sheet frequencies is used as a surrogate for fibrillar content. A β plaques were localized with a pan-amyloid fluorescent stain, and we found a relatively similar level of fibrils in all transgenic models (interquartile ranges of ratio were: 0.55–0.73 for APPPS1; 0.56–0.69 for ArcA β ; 0.59–0.78 for J20; 0.65–0.80 for TgF344). We cannot rule out that differences were masked by the variability of the measurements, arising from the various sectional plans which couldn't be precisely controlled with the fluorescent marker used here. However, the genetic AD patient had much higher levels of fibrils (interquartile ranges of ratio: 0.74–1.22) than the sporadic patient (0.54–0.60), probably due to the presence of more diffuse deposits in the sporadic case. Overall, these μ FTIR results showed that the fibrillar content of A β plaques is not the sole factor responsible for XPCT detection.

Multiple studies — based on X-ray fluorescence [26] or other methods like inductively coupled plasma mass spectrometry (ICP-MS) [50] — have linked AD pathogenesis with a disruption of the metal homeostasis in the brain, and more specifically copper, iron and zinc. These endogenous metals, whether in ionic or non-ionic forms [24], are found in neurons, and show a characteristic distribution in the different hippocampus areas [51]. In AD tissues, they are locally trapped in A β plaques at the expense of the surrounding tissue [21]. The metal concentrations we detected in the human A β plaques matched those from the seminal study by Lovell et al. [52]. As already observed, animal models had much lower metal quantities associated with A β plaques than humans [25, 53]. Importantly, copper, zinc and iron levels in the genetic patient were all significantly higher (2-fold increase) compared to the sporadic AD patient. In animals, A β plaques from each strain preferentially bound to

different metals (elevated Zn and Fe in J20; elevated Cu in TgF344). It has already been reported that iron is dysregulated within the hippocampus of J20 mouse model of AD [54]. Besides, some differences between mouse and rat hippocampus — particularly on copper accumulation [55] — may explain our results on copper-enriched plaques in the rat model compared to mouse strain.

Overall, the combination of XPCT, μ FTIR and μ XRF (performed on the same brains with advanced amyloid pathology) allows us to propose the following interpretations. In mouse models, A β plaques form numerous well-defined, and clearly visible deposits on XPCT images. Elevated levels of metals, mainly restricted to a small core area on XRF maps, are associated with a positive signal (hyperintensity) detectable in all three strains on XPCT. Importantly, the brighter signals (denser objects) are detected in the strain with the highest level of metals (J20). However, a larger dark rim (hypodensity) extending this core is also detected in APPPS1 and ArcA β , while absent in J20. A β fibrils, which match the fluorescence area and exceed the core area, cannot explain this difference as similar levels of fibrils were measured with μ FTIR. Other factors, such as structural [49] or proteomic [56] differences between strains, might play a role.

In the transgenic rat, the pathology develops more slowly. Few plaques, representing only a subset of the global amyloid burden, are indeed detected with XPCT, as small hyperdense spots. In contrast, amyloid fluorescence was widely detected in hippocampus, which pointed to a spatially widespread pathology (Supplemental Figure 2). In line with this, additional μ FTIR mapping experiments performed at ALBA synchrotron (beamline MIRAS) showed a global and unimodal increase in fibrillar content (Supplemental Figure 3 and associated Supplemental Methods), suggesting a diffuse accumulation of fibrils in the entire hippocampus. This implies that the few large fluorescent areas that were targeted in our μ FTIR/ μ XRF experiments are likely to be biased and maybe not be entirely representative of the pathology. Nevertheless, if we assume that the largest fluorescent plaques are the ones that give rise to XPCT detection, the high amounts of copper highlighted by μ XRF specifically in this model, are consistent with the results from mouse models, and could explain the hyperdensities, restricted to the core of A β plaques.

In humans, our data from two patients are obviously insufficient to disentangle the combined effect of β -sheet level and metal entrapment, which both drastically increased in the inherited case vs. sporadic one. However,

the strikingly diffuse pattern of metal entrapment observed in XRF (in comparison to rodent models, Figure 7) seems consistent with the detection of hyperdense plaques on XPCT images. More samples would be needed to understand the local density variations in different types of A β plaques (diffuse, neuritic) which could not be properly discriminated here. A direct comparison, at the individual plaque level, between 3D XPCT and subsequent 2D μ FTIR/ μ XRF would be ideal for that purpose, but would require cryogenic XPCT to avoid the chemical fixation used here (incompatible with μ XRF). Also, tau pathology, which is present in human samples in the form of neurofibrillary tangles (and potentially associated with microcalcifications [57]), but lacking in the rodent strains studied here, could contribute to the XPCT contrast, as it was recently shown in a triple-transgenic mouse model [11]. It is nevertheless remarkable that A β plaques from the genetic case are detectable in XPCT images, and this observation deserves further confirmation in other familial cases of AD.

In conclusion, XPCT has attracted increasing interest in neuroscience these last years for its ability to provide valuable microstructural characterization (myelin, vessels, neuron density, protein deposits) in excised, intact brain samples. XPCT can now be acquired in a few minutes at various resolutions, enabling a virtual dissection from the organ-level to the (sub-)cellular level. Indeed, high-resolution scans of whole organs are now possible, as recently demonstrated by the particularly impressive 3D imaging of a full human brain [58]. The present report reinforces the potential of XPCT to capture key aspects of the A β pathology. However, if XPCT is envisioned as a potential screening method for A β pathology in brain samples, it is crucial to understand which factors drive the detection of some and not all A β plaques. This study was specifically designed to tackle this question and highlighted sharp XPCT differences in A β pathology — which were related to the different levels of metals locally bound to A β plaques. This model-specific dyshomeostasis of endogenous metals might be a downstream consequence of the genetic alterations and could be region-specific or depend on the disease stage of progression. Additional factors, other than the overall β -sheet content, are likely to contribute to the XPCT-assessed density and remain to be identified.

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Authors' contributions (according to Contributor Role Taxonomy (CRediT))

- Conceptualization: FC
- Methodology: EB, SB, FC
- Resources: CS, TW, TD, DM, TB (APPPS1), JK (ArcA β), NR (J20), HB (TgF344), CO, EB, SB
- Investigation: MC, CS, TW, TD, CO, MW, EB, SB
- Formal analysis: MC, CS, SS, SB
- Data curation: MC, CS, SB
- Software:
- Visualization: MC, DM, FC
- Validation: MC
- Supervision: CS, EB, SB, FC
- Project administration: FC
- Funding acquisition: FC
- Writing – original draft: MC, FC
- Writing – review & editing: MC, DM, CS, TW, TD, JK, MW, EB, FC

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