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MICROGLIAL MOTILITY IN ALZHEIMER'S DISEASE AND AFTER Aβ42 IMMUNOTHERAPY: A HUMAN POST-MORTEM STUDY

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MICROGLIAL MOTILITY IN ALZHEIMER'S DISEASE AND AFTER Aβ42 IMMUNOTHERAPY: A HUMAN *POST-MORTEM* STUDY

SHORT RUNNING TITLE: Microglial motility in Alzheimer's disease

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ABSTRACT

Microglial function is highly dependent on cell motility, with baseline motility required for homeostatic surveillance activity and directed motility to migrate towards a source of injury. Experimental evidence suggests impaired microglial motility in Alzheimer's disease (AD) and therefore we have investigated whether the expression of proteins associated with motility is altered in AD and affected by the Aβ immunotherapy using *post-mortem* brain tissue of 32 controls, 44 AD cases, and 16 AD cases from our unique group of patients immunised against Aβ42 (iAD).

Sections of brain were immunolabelled and quantified for (i) the motility-related microglial proteins Iba1, cofilin 1 (CFL1), coronin-1a (CORO1A) and P2RY12, and (ii) pan-Aβ, Aβ42 and phosphorylated tau (ptau). The neuroinflammatory environment was characterised using Meso Scale Discovery multiplex assays. The expression of all four motility-related proteins was unmodified in AD compared with controls, whereas Iba1 and P2RY12, the homeostatic markers, were increased in the iAD group compared with AD. Iba1 and P2RY12 showed significant positive correlations with Aβ in controls but not in the AD or iAD groups. Pro- and anti-inflammatory proteins were increased in AD, whereas immunotherapy appears to result in a slightly less pro-inflammatory environment.

Our findings suggest that as Aβ appears during the ageing process, the homeostatic Iba1 and P2RY12 –positive microglia respond to Aβ, but this response is absent in AD. Aβ-immunisation promoted increased Iba1 and P2RY12 expression, likely reflecting increased baseline microglial motility but without restoring the profile observed in controls.

KEYWORDS: human microglia, cell motility, Alzheimer's disease, Aβ-immunotherapy, neuroinflammation

INTRODUCTION

Evidence from genetics, experimental and human *post-mortem* studies [47] supports the involvement of microglia, the resident immune cells of the brain, in the pathological cascade that leads to the development of Alzheimer's disease (AD). However, their role in the disease is still not fully understood [31]. In the healthy brain, microglia survey the parenchyma, constituting its first line of defence against pathogens or injury [10, 34], and interact with neurons helping to shape synaptic communication [29, 52-54, 56].

One of the most prominent characteristics of microglia is their motility, which is essential for the cells to perform their functions. Two types of motility are present in microglial cells: *baseline motility*, which consists of the extension, retraction, and movement of the microglial processes [18, 37], allowing microglia to survey their environment, clear cellular debris, interact with neurons and synapses, and remodel the extracellular matrix [34]. If, while performing this baseline motility, a microglial cell encounters molecules that could be indicative of infection or tissue damage, the cell will then switch to *directed motility*. Directed motility consists of a targeted extension of processes towards the source of injury [18]. At the molecular level, microglial motility depends on two mechanisms: i) reorganisation of the actin cytoskeleton and ii) purinergic signalling [13].

The actin cytoskeleton consists of a dynamic filament network, regulated by a complex machinery that involves several proteins. Some of the most important actin-related proteins in the microglial cytoskeleton are the coronins, the cofilins and the ionised calcium-binding adapter molecule (Iba1) [1, 46]. The coronin gene family contains six members with roles as regulators of actin dependent processes, such as cell motility and vesicle trafficking [40, 41]. Coronins localise to sites of dynamic actin assembly, regulating filament organisation via interactions between actin and the Arp2/3 protein complex. This results in the building of branches in the actin filament network [19]. Coronin 1a (CORO1A) plays a large role in the normal immune system as the most prominent coronin in cells of haematopoietic origin [12], and has been previously identified in human microglia [1]. Cofilins, and particularly cofilin 1 (CFL1), are actin-binding proteins that depolymerise and separate actin filaments [44]. Lastly, Iba1 is a cross-linking protein, the role of which is to organise actin filaments into networks. In microglia, Iba1 is crucial for the formation of actin bundles, [35], an event necessary for the construction of lamellipodia, filopodia, and membrane ruffles. These structures are essential for microglial migration and phagocytosis. Due to its relatively homogeneous distribution along the cell body and processes, Iba1 has been widely used as a marker to identify microglia in humans and experimental models. Purinergic signalling plays an important role in microglial motility. The presence of extracellular nucleosides (ATP, ADP, UTP, UDP), released as a

result of neuronal death, triggers the activation of P2Y receptors. The ADP receptor P2RY12 is now regarded as a microglia-specific marker, mainly associated with directed motility, driving chemotaxis through a mechanism that involves the activation of potassium (K^+) channels [26]. Therefore, both actin reorganisation and purinergic signalling are essential to the two forms of microglial motility.

Alterations of microglial motility have been documented in AD, with (i) microglial clusters identified around Aβ plaques indicating movement of microglia as part of their response to Aβ aggregation [16, 57], (ii) in AD mouse models, impairment of directed motility and phagocytic activity [23], and (iii) morphological changes in plaque-associated cells [38]. Aβ-immunotherapy is one of the major current therapeutic approaches being investigated in AD [51]. The first clinical trial of Aβ immunotherapy in humans involved active immunisation with a full-length synthetic Aβ42 compound named AN1792 [3]. Our neuropathological study of these patients has shown a markedly reduced plaque load associated with microglial changes [57, 58]. However, these changes were not associated with reduced cognitive decline [17, 33].

In this study, we have explored whether the expression of proteins associated with motility is altered in AD and affected by the Aβ immunotherapy using *post-mortem* brain tissue of controls, AD cases, and our unique group of AD cases immunised against Aβ42.

MATERIAL AND METHODS

Cases

Brain tissue from 76 donors was sourced from the South West Dementia Brain Bank comprising 44 AD cases and 32 controls. Alzheimer's cases had a clinical diagnosis of sporadic AD made during life and satisfied *post-mortem* neuropathological consensus criteria for AD [20]. Cases with any other significant brain pathologies such as stroke, tumour, or traumatic brain injury were excluded from the study. Controls were aged-matched cases, with no history of neurological or psychiatric disease or symptoms of cognitive impairment. Additionally, 16 immunised AD subjects (iAD) who participated in the AN1792 clinical trial were also included in the study. The characteristics of the groups are presented in Table 1 and individual details in the supplementary Tables 1, 2 and 3.

Cerebral cortex from the inferior parietal lobule was investigated in all cases. This is one of the areas recommended for neuropathological assessment by the CERAD (Consortium to Establish a registry for Alzheimer's Disease) [11] as it is significantly affected by AD pathology. Formalin-fixed paraffin embedded tissue was used for the immunodetection of neuropathological and neuroinflammatory markers. Frozen tissue, available for 31 controls, 35 AD cases and 11 iAD cases was used for detection of inflammation-related proteins by Meso Scale Discovery multiplex assays.

Immunohistochemistry

Immunohistochemistry was performed on 4μm paraffin-embedded sections, targeting the markers of AD pathology pan-Aβ (clone 4G8, BioLegend), Aβ42 (clone 21F12, Elan Pharmaceuticals Ltd) and the phosphorylated (p)tau (clone AT8, ThermoScientific) protein, as well as the microglial motility-related proteins Iba1 (rabbit polyclonal, Wako Chemicals), CFL1 (polyclonal rabbit, ThermoScientific), CORO1A (rabbit polyclonal, LifeSpan Biosciences) and P2RY12 (rabbit polyclonal, Sigma Aldrich). Bound antibodies were visualized using the avidin–biotin–peroxidase complex method (Vectastain Elite, Vector Laboratories) with 3,3′-diaminobenzidine as chromogen and 0.05% hydrogen peroxide as substrate (Vector Laboratories). All sections were counterstained with haematoxylin, then dehydrated and mounted in Pertex (Histolab Products AB). The staining was performed in two batches with each batch containing cases from all groups (Control, AD, iAD). All experiments included a negative control slide incubated in buffer with no primary antibody and a positive control slide containing a specific tissue type known to express the protein of interest (e.g. tonsil).

Quantification of staining

Quantification was blinded to the case designation and performed on the grey matter in the same sulcus of the inferior parietal lobule for all cases. For each antibody, the slides were scanned together at a magnification of x20 in an automated slide scanner microscope Olympus VS110 (Olympus America Inc.) and visualised with Olympus VS-Desktop software using a specialised add-in that allows the extraction of the region of interest (ROI) of 0.25mm^2. For each slide, 30 ROIs were selected in a zig-zag pattern within the grey matter, ensuring coverage of all cortical layers. Extracted ROIs were analysed with the ImageJ software (version 1.49u, Wayne Rasband, NIH, USA) using an automated macro specific to each antibody. The area fraction labelled by the antibody in each ROI was obtained by quantifying the presence or absence of the staining in each pixel, expressed as protein load (%).

Cytokine assay

Inflammatory proteins were measured on the V-Plex Meso Scale Discovery (MSD) electrochemiluminescence multi-spot assay platform (MesoScale Diagnostics, Rockville USA). 100 mg of frozen grey matter were homogenised at a tissue concentration of 20% w/v in RIPA lysis buffer (Thermo Fisher Scientific), supplemented with protease inhibitors (Sigma Aldrich) and phosphatase inhibitors (Thermo Fisher Scientific). Total protein concentration in the supernatant was measured by BCA Protein Assay Kit (Thermo Fisher Scientific). 25 μl of brain homogenate (1:2 dilution) were used for the V-PLEX human proinflammatory panel 1 and cytokine panel 1, and each plate was read on a Meso Quickplex SQ120 (Meso Scale

Discovery) according to manufacturer's instructions. Absolute target protein levels (pg/ml) were obtained and normalised with respect to the total protein concentration, as obtained by the BCA assay. Three frozen homogenates from a multiple sclerosis brain containing either chronic inactive, acute or chronic active lesions were included as positive controls.

Statistical analysis

Statistical analysis was performed using the IBM SPSS v24 statistical software package (SPSS Inc. Chicago IL). The normality of distribution of each marker across the groups was assessed by the Shapiro-Wilk test. Data were not normally distributed, and thus non-parametric tests were used. Comparisons among study groups were performed using the Kruskal-Wallis test. The Spearman's test was performed to assess correlations between variables – to explore the relationship (i) between microglial motility proteins and key features of AD pathology (Aβ and tau loads), and (ii) between the microglial-motility proteins. To account for multiple testing, the Benjamini-Hochberg procedure to control for the false discovery rate (FDR) was used as *post-hoc* correction. For all tests, an adjusted *P* value <0.05 was considered significant.

RESULTS

Aβ and tau pathology

Aβ and ptau immunoreactivity was quantified in order to characterise the cases with respect to AD pathology and to permit correlations with the microglial motility-related proteins. A significantly increased pan-Aβ load was observed in AD compared to control cases (*P*<0.001), whereas it was significantly decreased in the iAD group compared to AD cases (*P*<0.001), as expected. No significant difference was observed between control and iAD cases (Figure 1A). Aβ42 load was significantly increased in AD *vs* control cases (*P*=0.006) and decreased in iAD *vs* AD cases (*P*<0.001), as expected. Interestingly, Aβ42 load, was also significantly decreased in iAD compared to control cases (*P*=0.021) (Figure 1B). Ptau was significantly increased in AD *vs* control cases (*P*<0.001) and decreased in iAD *vs* AD cases (*P*=0.026), with no difference between the iAD and control groups (Figure 1C).

Microglial motility-related proteins

Immunohistochemistry for the four proteins related to microglial motility was investigated in the cortical grey matter. Iba1, CFL1 and CORO1A immunolabelled microglia and perivascular macrophages and in AD microglial clusters around amyloid plaques. While P2RY12 was specific to microglia with no labelling of perivascular macrophages (Figure 2).

Quantification of these four proteins showed no significant differences between AD and the control group: Iba1: control=0.34% (IQR: 0.08%-0.63%) *vs* AD=0.29%(IQR: 0.13%-0.68%), *P*=0.788; CFL1: control=0.15% (IQR: 0.03%-0.038%) *vs* AD=0.08% (IQR: 0.04%-0.022%), *P*=0.314; P2RY12: control=0.00% (IQR: 0.00%-0.55%) *vs* AD=0.09% (IQR: 0.00%-1.09%), *P*=0.290; CORO1A: control=0.21% (IQR: 0.10%-0.52%) *vs* AD=0.17% (IQR: 0.03%-0.33%), *P*=0.406. However, there was a significant increase for Iba1 and P2RY12 in iAD compared to AD cases: Iba1: AD=0.29% (IQR: 0.13%-0.68%) *vs* iAD=0.87% (IQR: 0.40%-1.11%), *P*=0.036; P2RY12: AD=0.09% (IQR: 0.00%-1.09%) *vs* iAD=0.60% (IQR: 0.50%-1.08%), *P*=0.041 (Figure 3). No significant difference was found in CFL1 and CORO1A loads between the AD and iAD groups: CFL1: AD=0.08% (IQR: 0.04%-0.022%) *vs* iAD 0.13% (IQR: 0.06%-0.41%), *P*=0.314; CORO1A: AD=0.17% (IQR: 0.03%-0.33%) *vs* iAD= 0.20% (IQR: 0.03%-0.61%), *P*=0.406).

We then explored whether the expression of the microglial motility-related proteins was correlated with the proteins that aggregate in AD. In the control group, correlations were detected for Iba1 and P2RY12 with (i) pan-Aβ (r_s=0.440, *P*=0.046 and r_s=0.443, *P*=0.042; respectively) and (ii) Aβ42 (r_s=0.691, *P*<0.001 and r_s=0.629, *P*<0.001; respectively) (Table 2). No correlations were observed with pan-Aβ and Aβ42 in the AD or iAD groups, and with ptau for the three groups.

We also assessed whether the different mechanisms involved in motility (actin-related or purinergic signalling) were related. In the control group, Iba1 was correlated with CFL1 (r_s=0.502, *P*=0.025) and P2RY12 (r_s=0.572, *P*=0.005). In the AD cases, Iba1 was related to CFL1 (r_s=0.559, *P*<0.001), CORO1A (r_s=0.436, *P*=0.014) and P2RY12 (r_s=0.558, *P*<0.001). CFL1 was also correlated with CORO1A (r_s=0.530, *P*=0.002) and P2RY12 (r_s=0.542, *P*=0.001), and CORO1A with P2RY12 (r_s=0.357; *P*=0.049). No correlation was detected in the iAD group.

Neuroinflammatory environment

To explore the relationship between microglial motility and the neuroinflammatory environment, the MSD platform was used to measure the levels of IFNγ, IL1β, IL2, IL4, IL6, IL8, IL10, IL12p70, IL13, TNFα, IL1α, IL5, IL7, IL12/IL23p40, IL15, IL16, IL17A, GM-CSF, TNFβ and VEGF in the three groups. Significant increases in the AD group compared to the control group were observed for: IFNγ (*P*<0.001), IL10 (*P*=0.003), IL12p70 (*P*<0.001), IL13 (*P*<0.001), IL2 (*P*=0.002), IL4 (*P*=0.007), TNFα (*P*<0.001), IL15 (*P*=0.024), IL16 (*P*=0.020) and TNFβ (*P*=0.009). In the iAD group compared to AD, IL13 (*P*=0.017), IL8 (*P*=0.023) and VEGF (*P*<0.001) were significantly increased; whereas IL7 was significantly decreased (*P*=0.012). Of note, of the proteins that were significantly different between AD and iAD, IL13

and VEGF were also significantly increased with respect to the control levels (IL13: $P<0.001$; VEGF: $P=0.012$), while IL7 was significantly decreased ($P=0.003$) (Table 4).

DISCUSSION

Evidence from animal studies shows that microglia are highly motile cells [14, 16, 34, 49], with motility impaired in AD models [6, 23]. This highlights motility as a functional aspect of microglia relevant to disease pathogenesis. Our aim was to characterise the expression of motility-related microglial proteins in the human brain and to evaluate its changes in AD and after Aβ immunotherapy. In the present study, we observed increased expression of the microglial homeostatic markers Iba1 and P2RY12 in the immunised group, which was associated with a less pro-inflammatory environment than observed in AD. We acknowledge that this approach has limitations, partly inherent to any *post-mortem* study, which reside in the absence of exploration of the temporal relationship between the different markers investigated, and with the analysis limited to the late-stage consequences of AD. Additionally, other proteins such as actin, the Arp2/3 complex, or the integrins also relevant for motility have not been evaluated here, as our investigation focuses on microglial-specific motility-related proteins. The strength of the study is in the examination of AD itself, rather than an experimental model of the disease which does not represent the whole extent of the complexity of the human disease. Specifically, most rodent models lack tau pathology, the generation and propagation of which microglia are proposed to have an important role. The novelty of our study resides in the combined quantitative assessment of several markers associated with microglial motility in relation to the neuroinflammatory environment, and the accumulation of Aβ and ptau. This study also takes advantage of the exceptional chance to explore the effect of Aβ42-immunotherapy which constitutes an important topic of study since there is currently substantial resource being invested in this therapeutic approach.

Aβ and tau pathology

As expected, the expression of the key features of AD neuropathology, Aβ and ptau, was increased in AD. The effect of the immunotherapy on AD has been previously reported by us [4, 32, 33, 42]. However in this study, we were able to compare the effects of the treatment with both controls and unimmunised AD. Interestingly, we observed that Aβ42 immunotherapy reduced the overall Aβ levels to those of controls, indicating an effect not restricted to the Aβ42 form. This is consistent with our studies on the same immunised cohort using antibodies specific for the Aβ40, Aβ42 and Aβ43 species and reporting significant decrease of the three species [21, 32].

Of note, its effect on the Aβ42 form seems to be more pronounced, with a reduction to significantly lower levels than those of controls. This highlights a possible higher affinity of the immunisation towards the Aβ42 specie, perhaps due to the fact that full-length Aβ42 was the immunogen originally used in the AN1792 trial, even though earlier studies showed that the antibodies produced by the patients were primarily N-terminal specific [24]. This implies that the antibodies are able to target all different species of Aβ. The ptau load was reduced to the level of the controls, confirming the effect of Aβ-immunotherapy on the tau pathology [4, 33]. Indeed, our previous studies on the immunised cohort showed that ptau load was reduced after Aβ immunisation, in association with a lower expression of the tau phosphorylating enzyme GSK-3b [2]. Ptau load is mainly constituted of tau-containing dystrophic neurite clusters and ptau in neuronal processes [4], rather than tangles. Nevertheless, despite the decrease in ptau load as assessed by quantification in specific localised neuroanatomical regions of cortex, most of the iAD subjects still had progressed to Braak stages V or VI, as evaluated by the anatomical distribution of neurofibrillary tangles throughout the brain areas. We interpret these findings as indicating that localised removal of Aβ following Aβ immunotherapy results in a corresponding removal of ptau in that location, consistent with the amyloid cascade hypothesis. However, it does not stop the spread of ptau through the brain, consistent with the evidence for prion-like spread of ptau [4, 33].

Microglial motility-related proteins

In AD, expression of all four motility-related proteins examined was not modified compared with controls, consistent with another *post-mortem* AD study [45]. In this paper, they evaluated expression of Iba1 and P2RY12 and also found no difference in the expression of both proteins between the control and AD groups. Interestingly, in that study, when the AD cases were split according to the Braak stage, a decrease for both Iba1 and P2RY12 proteins was observed in the late-stage of the disease (Braak stage V/VI) but only in the hippocampal area, a region heavily affected by neuronal loss and tau accumulation [50]. In our study, even though most of the AD cases correspond to Braak stages V and VI, we did not observe decrease in these proteins, which could be indicative of regional differences between the hippocampus and the cerebral cortex due to the exacerbated degree of neuronal death and tau aggregation in the hippocampal region in late stages of the disease. With respect to CFL1, our observation confirms previous reports of no difference in CFL1 expression in AD [39]. The presence of CORO1A has been observed in human microglia [1], but to our knowledge has never been assessed in AD.

After Aβ immunotherapy in AD, a 3-fold increase in Iba1 expression and a 6.6-fold increase in P2RY12 expression was observed. Data on anti-Aβ antibody titres measured in

the blood during life some years earlier were available [57] and did not correlate with the microglial analyses. Iba1 is expressed by microglia in both physiological and disease conditions, suggesting that Iba1 participates in both baseline and directed motility. *In vitro* studies have characterised the role of Iba1 in relation to membrane ruffling, and the formation of phagocytic cups is Iba1-dependent [35]. Therefore, the higher Iba1 expression observed is in accordance with the reported increased phagocytic activity associated with Aβ clearance [58] as the consequence of immunotherapy stimulating microglial phagocytosis towards Aβ. Thus, after immunotherapy, Iba1 expression is likely to reflect the motility changes associated with phagocytosis. Similarly to Iba1, P2RY12 is detected on all microglial cells and a role of P2RY12 in directed motility is also well-documented. The release of ATP to the extracellular space, caused by cell death, and its subsequent hydrolysis to ADP activates P2RY12 [48]. Studies have shown that microglia from mice lacking P2RY12 expression show normal baseline motility but impaired directed motility [15, 26]. Moreover, it was observed that P2RY12 expression in ramified microglia was markedly decreased as cells turn to the reactive/amoeboid form [15, 38]. This suggests a role of P2RY12 in directed motility taking place at an early stage of the cell activation. Thus, P2RY12 expression after immunotherapy might suggest an increase both in baseline and directed motility. This is also consistent with the neuroinflammatory environment observed in the immunised group, which is slightly leaning towards a more anti-inflammatory profile, considering evidence that P2RY12 is downregulated under pro-inflammatory conditions and upregulated in an anti-inflammatory context [22, 30]. Of note, a previous study by our group in the immunised group showed that neuronal loss is correlated with Iba1-positive microglia and phagocytic CD68-positive microglia associated with pro-apoptotic neurons, but without accelerated cognitive decline compared to non-immunised AD patients [36]. This suggests that immunotherapy may enhance microglial elimination of damaged and dysfunctional neurons. Considering the role of P2RY12 as a purinergic receptor upregulated with the release of ATP that occurs during cell damage, it could be hypothesized that the increased P2RY12 expression in the immunised cohort reflects microglial increased response, as a result of the treatment, towards damaged neurons.

Motility-related proteins and the pathology

Relationships between the microglial motility-related proteins and Aβ and ptau, key pathological features of AD, were explored. In controls, Iba1 and P2RY12 were associated with Aβ, with these associations lost in AD and not restored by the immunotherapy. It is recognised that with ageing, Aβ accumulates independently of the dementia status [8]. Therefore, the relationship of Iba1 and P2RY12 with Aβ in the absence of dementia may reflect a healthy chemotactic microglial response towards Aβ, via the directed motility mechanism, as previously reported in the Cognitive Function and Ageing Studies group [28]. In AD, these

correlations were lost, possibly due to either (i) Aβ over-accumulation to a level at which microglia are overwhelmed and no longer capable of responding accordingly, (ii) microglia attracted to plaques and been immobilised there [57], or (iii) microglia being “distracted” by the tau pathology [28]. However, the absence of correlation of the motility-related proteins with Aβ or ptau in the AD group is in favour of a microglial response towards other pathological changes, such as the synaptic/neuronal loss. Of note, the correlations were not restored after immunotherapy despite Aβ levels as lower as in the controls and this despite the increased Iba1 and P2RY12 expression. No relationship was observed with CFL1 or CORO1A.

Since no correlations were found between the microglial motility-related markers and ptau, this implies that the motility response driven by the proteins studied is independent of tau, or at least they do not directly interact with tau.

Interactions between motility-related proteins

Correlations between the microglial motility-related proteins were also investigated. In controls, Iba1 correlated with CFL1 and P2RY12, reflecting an interaction between the actin-related motility mechanisms and the purinergic signalling pathway in physiological conditions. Interestingly, in AD all markers were related to each other, implying that the pathogenesis elicited a coordinated response of the microglial motility proteins as part of the pathogenesis spreading. In the immunised group, no relation was observed despite the increased expression of the homeostatic markers Iba1 and P2RY12. This implies that immunotherapy did not restore the physiological profile of microglial protein interactions.

Neuroinflammatory environment

Inflammation-related proteins were evaluated to characterise the microenvironment. In AD, 10 were increased, including the pro-inflammatory proteins IFNγ, TNFα, TNFβ, IL12p70, IL2, IL15 and IL16, and the anti-inflammatory proteins IL4, IL10 and IL13. This reflects that both pathways of activation coexist in human AD as previously reported [9, 55].

After Aβ immunotherapy, only four markers were modified when compared with unimmunised AD, with increased expression of the pro-inflammatory chemokine IL8, the anti-inflammatory cytokine IL13, the vascular endothelial growth factor (VEGF), and a decrease in the pro-inflammatory cytokine IL7. IL13 and IL7 expression tends to reflect a slightly less pro--inflammatory environment after the treatment. VEGF expression was the most affected by immunotherapy with a 5-fold increase. This might be the result of (i) increased soluble Aβ species following plaque solubilisation [25] leading to neurotoxicity and angiogenesis via a VEGF-mediated mechanism [7], or (ii) reflected the vascular side effects reported after immunotherapy, known as amyloid-related imaging abnormalities (ARIA) including microhaemorrhages and oedema [5, 43]. Interestingly, both VEGF and IL8 are involved in

angiogenesis [27]. It is worth noting that, from the four markers that differed between AD and controls, IL13 and VEGF were also significantly increased with respect to controls, while IL7 was decreased with respect to control, emphasizing that Aβ-immunotherapy, even though inducing a slightly more anti-inflammatory environment, did not restore the normal state.

CONCLUSION

In conclusion, our study supports the experimental observation that microglial motility is impaired in AD, as evidenced by the changes observed in the interactions of the motility-related proteins with each other and with Aβ. As part of the AD pathogenic process, microglia move from baseline to directed-motility, involving all investigated proteins (Iba1, CFL1, CORO1A and P2RY12). Aβ immunotherapy seemed to result in increased microglial baseline motility (suggested by increased Iba1 and P2RY12 expression, which are considered "homeostatic" microglial markers), potentially due to decreased Aβ and/or tau, but without restoration of the physiological status of microglia.

LIST OF ABBREVIATIONS

Aβ	Amyloid-β
AD	Alzheimer's disease
iAD	immunised AD
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ARIA	Amyloid-related imaging abnormalities
BCA	Bicinchoninic acid assay
CFL1	Cofilin
CORO1A	Coronin 1A
GSK	Glycogen Synthase Kinase
Iba1	Ionized calcium-binding adaptor molecule 1
IFN	Interferon
IL	Interleukin
ICAM	Intercellular adhesion molecule
IQR	Interquartile range
MSD	MesoScale Discovery
P2RY12	Purinergic receptor P2Y12

ptau	Hyperphosphorylated tau
ROI	Region of interest
TGF	Transforming growth factor
TNF	Tumour necrosis factor
UDP	Uridine diphosphate
UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor

DECLARATIONS

Ethics approval: The study was covered by the following ethical approvals: (i) the South West Dementia Brain bank (NRES Committee South West Central Bristol, REC reference: 08/H0106/28+5 for the controls and AD cases; (ii) the Southampton and South West Hampshire Local Research Ethics Committees (reference: LRC 075/03/w) for the iAD cases; (iii) The Multiple Sclerosis Society of Great Britain and Northern Ireland and Parkinson's UK (reference 08/MRE09/31+5) for frozen tissue used as positive controls for the cytokine assay.

Consent for publication: Not applicable

Availability of data and material: The data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no conflict of interest.

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Authors' contribution: DKFB immunolabelled the cases for the different motility related and neuropathological proteins and prepared the samples for the cytokine assays. DKFB and BG performed protein quantification. LCL performed the multiplex assays. DKFB collected all data and with DB analysed and interpreted them. CH and JARN advised on the clinical and neuropathological relevance of the findings, respectively. JARN and DB conceived and

designed the study and DKFB and DB wrote the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1 Ahmed Z, Shaw G, Sharma VP, Yang C, McGowan E, Dickson DW (2007) Actin-binding proteins coronin-1a and IBA-1 are effective microglial markers for immunohistochemistry. J Histochem Cytochem 55: 687-700 Doi 10.1369/jhc.6A7156.2007

2 Amin J, Paquet C, Baker A, Asuni AA, Love S, Holmes C, Hugon J, Nicoll JA, Boche D (2015) Effect of amyloid-beta (Abeta) immunization on hyperphosphorylated tau: a potential role for glycogen synthase kinase (GSK)-3beta. Neuropathol Appl Neurobiol 41: 445-457 Doi 10.1111/nan.12205

3 Bayer AJ, Bullock R, Jones RW, Wilkinson D, Paterson KR, Jenkins L, Millais SB, Donoghue S (2005) Evaluation of the safety and immunogenicity of synthetic Abeta42 (AN1792) in patients with AD. Neurology 64: 94-101.

4 Boche D, Donald J, Love S, Harris S, Neal JW, Holmes C, Nicoll JA (2010) Reduction of aggregated Tau in neuronal processes but not in the cell bodies after Abeta42 immunisation in Alzheimer's disease. Acta Neuropathol 120: 13-20 Doi 10.1007/s00401-010-0705-y

5 Boche D, Zotova E, Weller RO, Love S, Neal JW, Pickering RM, Wilkinson D, Holmes C, Nicoll JA (2008) Consequence of Abeta immunization on the vasculature of human Alzheimer's disease brain. Brain 131: 3299-3310

6 Bolmont T, Haiss F, Eicke D, Radde R, Mathis CA, Klunk WE, Kohsaka S, Jucker M, Calhoun ME (2008) Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. J Neurosci 28: 4283-4292 Doi 28/16/4283

7 Cameron DJ, Galvin C, Alkam T, Sidhu H, Ellison J, Luna S, Ethell DW (2012) Alzheimer's-related peptide amyloid-beta plays a conserved role in angiogenesis. PLoS One 7: e39598 Doi 10.1371/journal.pone.0039598

8 CFAS (2001) Pathological correlates of late-onset dementia in a multicentre, community-based population in England and Wales. Neuropathology Group of the Medical Research Council Cognitive Function and Ageing Study Lancet 357: 169-175

9 Colton CA, Mott RT, Sharpe H, Xu Q, Van Nostrand WE, Vitek MP (2006) Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. J Neuroinflammation 3: 27-39 Doi 1742-2094-3-27

10 Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB (2005) ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 8: 752-758 Doi 10.1038/nn1472

11 Fillenbaum GG, van Belle G, Morris JC, Mohs RC, Mirra SS, Davis PC, Tariot PN, Silverman JM, Clark CM, Welsh-Bohmer KAet al (2008) Consortium to Establish a Registry for Alzheimer's Disease (CERAD): The first twenty years. Alzheimer's & Dementia 4: 96-109
12 Foger N, Rangell L, Danilenko DM, Chan AC (2006) Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. Science 313: 839-842 Doi 10.1126/science.1130563
13 Franco-Bocanegra DK, McAuley C, Nicoll JAR, Boche D (2019) Molecular Mechanisms of Microglial Motility: Changes in Ageing and Alzheimer's Disease. Cells 8: Doi 10.3390/cells8060639
14 Gyoneva S, Davalos D, Biswas D, Swanger SA, Garnier-Amblard E, Loth F, Akassoglou K, Traynelis SF (2014) Systemic inflammation regulates microglial responses to tissue damage in vivo. Glia 62: 1345-1360 Doi 10.1002/glia.22686
15 Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D (2006) The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat Neurosci 9: 1512-1519 Doi 10.1038/nn1805
16 Hefendehl JK, Neher JJ, Suhs RB, Kohsaka S, Skodras A, Jucker M (2014) Homeostatic and injury-induced microglia behavior in the aging brain. Aging Cell 13: 60-69 Doi 10.1111/acel.12149
17 Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, Bayer A, Jones RW, Bullock R, Love S, Neal JWet al (2008) Long-term effects of Abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. Lancet 372: 216-223 Doi 10.1016/S0140-6736(08)61075-2
18 Hristovska I, Pascual O (2015) Deciphering Resting Microglial Morphology and Process Motility from a Synaptic Prospect. Front Integr Neurosci 9: 73 Doi 10.3389/fnint.2015.00073
19 Humphries CL, Balcer HI, D'Agostino JL, Winsor B, Drubin DG, Barnes G, Andrews BJ, Goode BL (2002) Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin. J Cell Biol 159: 993-1004 Doi 10.1083/jcb.200206113
20 Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, Dickson DW, Duyckaerts C, Frosch MP, Masliah Eet al (2012) National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. Alzheimers Dement 8: 1-13 Doi 10.1016/j.jalz.2011.10.007
21 Jakel L, Boche D, Nicoll JAR, Verbeek MM (2019) Abeta43 in human Alzheimer's disease: effects of active Abeta42 immunization. Acta Neuropathol Commun 7: 141 Doi 10.1186/s40478-019-0791-6
22 Klein B, Mrowetz H, Barker CM, Lange S, Rivera FJ, Aigner L (2018) Age Influences Microglial Activation After Cuprizone-Induced Demyelination. Front Aging Neurosci 10: 278 Doi 10.3389/fnagi.2018.00278
23 Krabbe G, Halle A, Matyash V, Rinnenthal JL, Eom GD, Bernhardt U, Miller KR, Prokop S, Kettenmann H, Heppner FL (2013) Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. PLoS One 8: e60921 Doi 10.1371/journal.pone.0060921
24 Lee M, Bard F, Johnson-Wood K, Lee C, Hu K, Griffith SG, Black RS, Schenk D, Seubert P (2005) Abeta42 immunization in Alzheimer's disease generates Abeta N-terminal antibodies. Ann Neurol 58: 430-435
25 Maarouf CL, Daugs ID, Kokjohn TA, Kalback WM, Patton RL, Luehrs DC, Masliah E, Nicoll JA, Sabbagh MN, Beach TGet al (2010) The biochemical aftermath of anti-amyloid immunotherapy. Mol Neurodegener 5: 39 Doi 1750-1326-5-39
26 Madry C, Arancibia-Carcamo IL, Kyrargyri V, Chan VTT, Hamilton NB, Attwell D (2018) Effects of the ecto-ATPase apyrase on microglial ramification and surveillance reflect cell depolarization, not ATP depletion. Proc Natl Acad Sci U S A 115: E1608-E1617 Doi 10.1073/pnas.1715354115
27 Martin D, Galisteo R, Gutkind JS (2009) CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial

cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. J Biol Chem 284: 6038-6042 Doi 10.1074/jbc.C800207200

28 Minett T, Classey J, Matthews FE, Fahrenhold M, Taga M, Brayne C, Ince PG, Nicoll JA, Boche D (2016) Microglial immunophenotype in dementia with Alzheimer's pathology. J Neuroinflam 13: 135-145 Doi 10.1186/s12974-016-0601-z

29 Miyamoto A, Wake H, Moorhouse AJ, Nabekura J (2013) Microglia and synapse interactions: fine tuning neural circuits and candidate molecules. Front Cell Neurosci 7: 70 Doi 10.3389/fncel.2013.00070

30 Moore CS, Ase AR, Kinsara A, Rao VT, Michell-Robinson M, Leong SY, Butovsky O, Ludwin SK, Seguela P, Bar-Or Aet al (2015) P2Y12 expression and function in alternatively activated human microglia. Neurol Neuroimmunol Neuroinflamm 2: e80 Doi 10.1212/nxi.0000000000000080

31 Mosher KI, Wyss-Coray T (2014) Microglial dysfunction in brain aging and Alzheimer's disease. Biochem Pharmacol 88: 594-604 Doi 10.1016/j.bcp.2014.01.008

32 Nicoll JA, Barton E, Boche D, Neal JW, Ferrer I, Thompson P, Vlachouli C, Wilkinson D, Bayer A, Games Det al (2006) Abeta Species Removal After Abeta42 Immunization. J Neuropathol Exp Neurol 65: 1040-1048

33 Nicoll JAR, Buckland GR, Harrison CH, Page A, Harris S, Love S, Neal JW, Holmes C, Boche D (2019) Persistent neuropathological effects 14 years following amyloid-beta immunization in Alzheimer's disease. Brain 142: 2113-2126 Doi 10.1093/brain/awz142

34 Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308: 1314-1318 Doi 1110647 [pii] 10.1126/science.1110647

35 Ohsawa K, Imai Y, Kanazawa H, Sasaki Y, Kohsaka S (2000) Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia. J Cell Sci 113 (Pt 17): 3073-3084

36 Paquet C, Amin J, Mouton-Liger F, Nasser M, Love S, Gray F, Pickering RM, Nicoll JA, Holmes C, Hugon Jet al (2015) Effect of active Abeta immunotherapy on neurons in human Alzheimer's disease. J Pathol 235: 721-730 Doi 10.1002/path.4491

37 Parkhurst CN, Gan W-B (2010) Microglia dynamics and function in the CNS. Current opinion in neurobiology 20: 595-600 Doi 10.1016/j.conb.2010.07.002

38 Plescher M, Seifert G, Hansen JN, Bedner P, Steinhauser C, Halle A (2018) Plaque-dependent morphological and electrophysiological heterogeneity of microglia in an Alzheimer's disease mouse model. Glia 66: 1464-1480 Doi 10.1002/glia.23318

39 Rahman T, Davies DS, Tannenberg RK, Fok S, Shepherd C, Dodd PR, Cullen KM, Goldsbury C (2014) Cofilin rods and aggregates concur with tau pathology and the development of Alzheimer's disease. J Alzheimers Dis 42: 1443-1460 Doi 10.3233/jad-140393

40 Roadcap DW, Clemen CS, Bear JE (2008) The role of mammalian coronins in development and disease. Subcell Biochem 48: 124-135 Doi 10.1007/978-0-387-09595-0_12

41 Rybakin V, Clemen CS (2005) Coronin proteins as multifunctional regulators of the cytoskeleton and membrane trafficking. Bioessays 27: 625-632 Doi 10.1002/bies.20235

42 Sakai K, Boche D, Carare R, Johnston D, Holmes C, Love S, Nicoll JA (2014) Abeta immunotherapy for Alzheimer's disease: effects on apoE and cerebral vasculopathy. Acta Neuropathol: Doi 10.1007/s00401-014-1340-9

43 Salloway S, Sperling R, Fox NC, Blennow K, Klunk W, Raskind M, Sabbagh M, Honig LS, Porsteinsson AP, Ferris Set al (2014) Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. N Engl J Med 370: 322-333 Doi 10.1056/NEJMoa1304839

44 Samstag Y, John I, Wabnitz GH (2013) Cofilin: a redox sensitive mediator of actin dynamics during T-cell activation and migration. Immunol Rev 256: 30-47 Doi 10.1111/imr.12115

45 Sanchez-Mejias E, Navarro V, Jimenez S, Sanchez-Mico M, Sanchez-Varo R, Nunez-Diaz C, Trujillo-Estrada L, Davila JC, Vizuete M, Gutierrez Aet al (2016) Soluble phospho-

tau from Alzheimer's disease hippocampus drives microglial degeneration. Acta Neuropathol 132: 897-916 Doi 10.1007/s00401-016-1630-5
46 Sasaki Y, Ohsawa K, Kanazawa H, Kohsaka S, Imai Y (2001) Iba1 is an actin-cross-linking protein in macrophages/microglia. Biochem Biophys Res Commun 286: 292-297 Doi 10.1006/bbrc.2001.5388
47 Song WM, Colonna M (2018) The identity and function of microglia in neurodegeneration. Nat Immunol 19: 1048-1058 Doi 10.1038/s41590-018-0212-1
48 Soulet C, Sauzeau V, Plantavid M, Herbert JM, Pacaud P, Payrastre B, Savi P (2004) Gi-dependent and -independent mechanisms downstream of the P2Y12 ADP-receptor. J Thromb Haemost 2: 135-146
49 Stence N, Waite M, Dailey ME (2001) Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. Glia 33: 256-266 Doi 10.1002/1098-1136(200103)33:3<256::AID-GLIA1024>3.0.CO;2-J [pii]
50 Stutzbach LD, Xie SX, Naj AC, Albin R, Gilman S, Lee VM, Trojanowski JQ, Devlin B, Schellenberg GD (2013) The unfolded protein response is activated in disease-affected brain regions in progressive supranuclear palsy and Alzheimer's disease. Acta Neuropathol Commun 1: 31 Doi 10.1186/2051-5960-1-31
51 Sumner IL, Edwards RA, Asuni AA, Teeling JL (2018) Antibody Engineering for Optimized Immunotherapy in Alzheimer's Disease. Front Neurosci 12: 254 Doi 10.3389/fnins.2018.00254
52 Tremblay ME, Lowery RL, Majewska AK (2010) Microglial interactions with synapses are modulated by visual experience. PLoS Biol 8: e1000527 Doi 10.1371/journal.pbio.1000527
53 Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29: 3974-3980 Doi 10.1523/JNEUROSCI.4363-08.2009
54 Weinhard L, di Bartolomei G, Bolasco G, Machado P, Schieber NL, Neniskyte U, Exiga M, Vadisiute A, Raggioli A, Schertel Aet al (2018) Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction. Nat Commun 9: 1228 Doi 10.1038/s41467-018-03566-5
55 Wilcock DM, Zhao Q, Morgan D, Gordon MN, Everhart A, Wilson JG, Lee JE, Colton CA (2011) Diverse inflammatory responses in transgenic mouse models of Alzheimer's disease and the effect of immunotherapy on these responses. ASN Neuro 3: 249-258 Doi 10.1042/AN20110018
56 Wu Y, Dissing-Olesen L, MacVicar BA, Stevens B (2015) Microglia: Dynamic Mediators of Synapse Development and Plasticity. Trends Immunol 36: 605-613 Doi 10.1016/j.it.2015.08.008
57 Zotova E, Bharambe V, Cheaveau M, Morgan W, Holmes C, Harris S, Neal JW, Love S, Nicoll JA, Boche D (2013) Inflammatory components in human Alzheimer's disease and after active amyloid-beta42 immunization. Brain 136: 2677-2696 Doi 10.1093/brain/awt210
58 Zotova E, Holmes C, Johnston D, Neal JW, Nicoll JA, Boche D (2011) Microglial alterations in human Alzheimer's disease following Abeta42 immunization. Neuropathol Appl Neurobiol 37: 513-524 Doi 10.1111/j.1365-2990.2010.01156.x

LEGENDS

Figure 1: Illustration and quantification of pan-Aβ, Aβ42 and ptau loads for the 3 groups. (A) We observe a significantly increased pan-Aβ load in AD *vs.* controls (*P*<0.001), whereas it is significantly decreased in iAD *vs.* AD (*P*<0.001). (B) Aβ42 quantification shows similar expression than pan-Aβ, with also a significantly decreased load in iAD *vs.* control groups (*P*=0.021). (C) Phosphorylated (p)tau shows a significantly increased load in AD *vs*. control (*P*<0.001) while it is decreased in iAD *vs*. AD groups (*P*=0.026). Counterstaining: Haematoxylin. Scale bar = 50 μm.

Figure 2: Illustration of the immunostaining obtained with the different microglial motility-related proteins. Counterstaining: Haematoxylin. Scale bar = 50 μm.

Figure 3: Quantification of the microglial motility-related proteins in the three groups. No difference was observed between the AD and control groups for the four proteins. Significantly increased Iba1 and P2RY12 loads were detected in the iAD *vs.* AD groups only (*P*=0.036 and *P*=0.041, respectively).

Table 1 - Demographic, clinical and *post-mortem* characteristics of the three groups

Groups		**Control n=32**	**AD n=44**	**iAD n=16**
Gender		17F:15M	28F:16M	7F:9M
Age of Death (years, mean±SD)		84±7	80±6	79±8
Age of AD onset (years, mean±SD)		n/a	70±7	67±8
Duration of AD (years, mean±SD)		n/a	10±3	12±4
Braak Stage		0-II: 29 III-IV: 3 V-VI: 0	0-II: 0 III-IV: 4 V-VI: 40	0-II: 0 III-IV: 1 V-VI: 15
***APOE* genotype**	*ε4/-*	3/28 (10.7%)	13/38 (34.2%)	6/10 (60.0%)
	ε4/ε4	1/28 (3.6%)	9/38 (23.7%)	3/10 (30.0%)
***Post-mortem* delay (hours, mean±SD)**		42 ± 23	42 ± 26	22 ± 25

Control neurologically/cognitively normal controls, *AD* Alzheimer's disease cases, *iAD* immunised Alzheimer's disease cases
F female, *M* male,
APOE genotyping was not available for all cases
n/a not-applicable, *SD* standard deviation

Table 2. Correlation of motility-related markers with pan-Aβ, Aβ42 and ptau

		Iba1	CFL1	P2RY12	CORO1A
pan-Aβ	**Control**	*r_s=0.440**	r_s=0.301	*r_s=0.443**	r_s=0.055
	AD	r_s=-0.055	r_s=0.077	r_s=0.219	r_s=0.009
	iAD	r_s=-0.143	r_s=0.011	r_s=0.054	r_s=-0.029
Aβ42	**Control**	*r_s=0.691****	r_s=0.459	*r_s=0.629****	r_s=0.051
	AD	r_s=-0.021	r_s=-0.172	r_s=0.078	r_s=-0.158
	iAD	r_s=-0.464	r_s=-0.575	r_s=0.171	r_s=0.464
ptau	**Control**	rs=-0.113	rs=-0.165	rs=0.017	rs=0.-173
	AD	rs=-0.239	rs=-0.043	rs=-0.249	rs=-0.019
	iAD	rs=-0.709	rs=-0.118	rs=0.191	rs=0.036

r_s Spearman's rank correlation, *P<0.05; *** P<0.001, significant P values are in italic.
CFL1 cofilin 1, *CORO1A* coronin-1A
Control neurologically/cognitively normal controls, *AD* Alzheimer's disease cases, *iAD* immunised Alzheimer's disease cases, *ptau*: phosphorylated tau

Table 3. Correlations between the motility-related markers

	Control	AD	iAD
Iba1 *vs*. CFL1	*r_S=0.502***	*r_S=0.559****	r_S=0.443
Iba1 *vs*. CORO1A	r_S=0.357	*r_S=0.436***	r_S=0.361
Iba1 *vs*. P2RY12	*r_S=0.572***	*r_S=0.558****	r_S=0.114
CFL1 *vs*. CORO1A	r_S=0.376	*r_S=0.530***	r_S=0.412
CFL1 *vs*. P2RY12	r_S=0.223	*r_S=0.542****	r_S=0.068
CORO1A *vs*. P2RY12	r_S=0.128	*r_S=0.357**	r_S=0.232

r_S Spearman's rank correlation; *P<0.05; **P<0.01; ***P<0.001; significant P values are in italic.
CFL1 cofilin 1, *CORO1A* coronin-1A
Control neurologically/cognitively normal controls, *AD* Alzheimer's disease cases, *iAD* immunised Alzheimer's disease cases

Figure 1

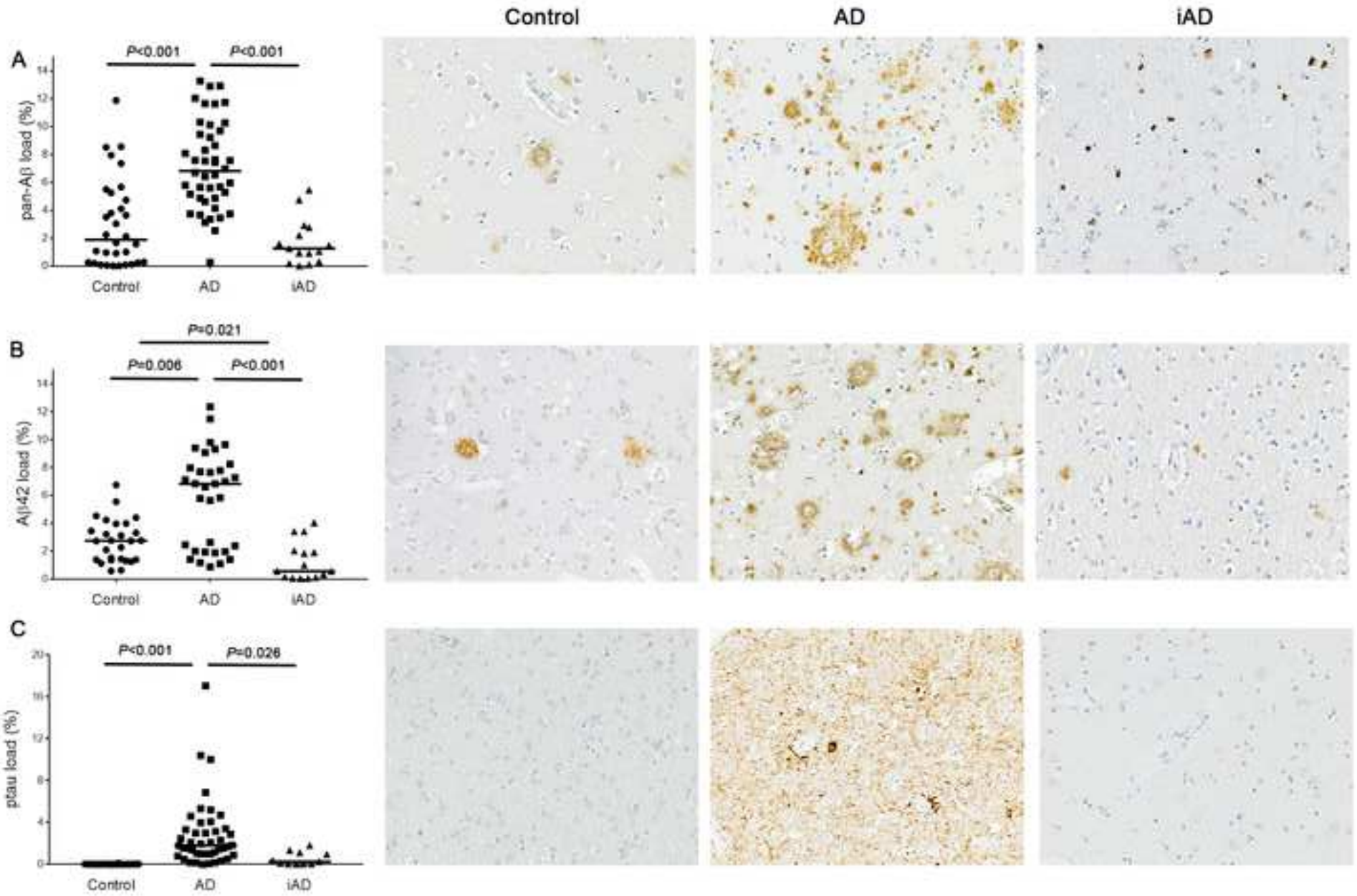

Figure 2

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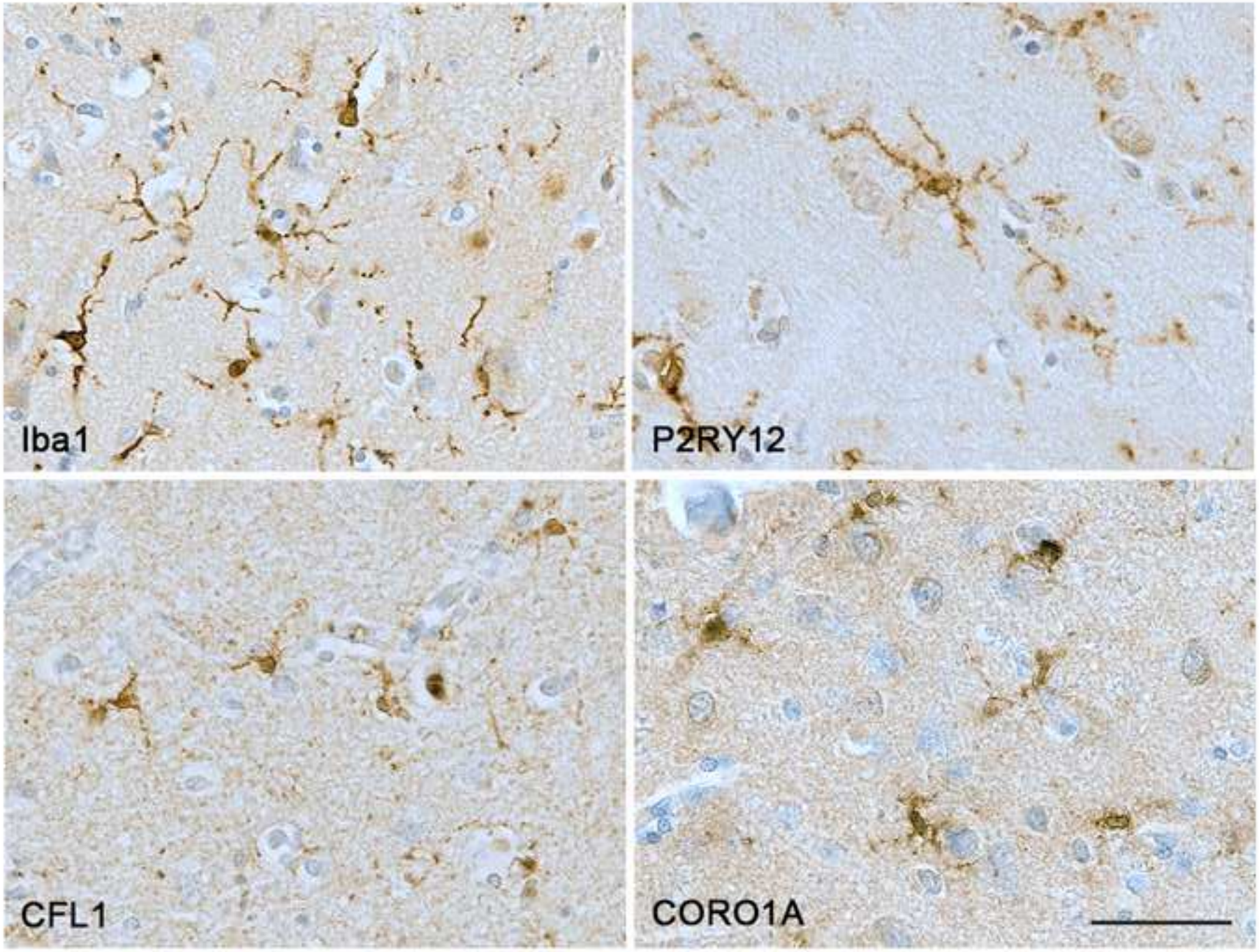
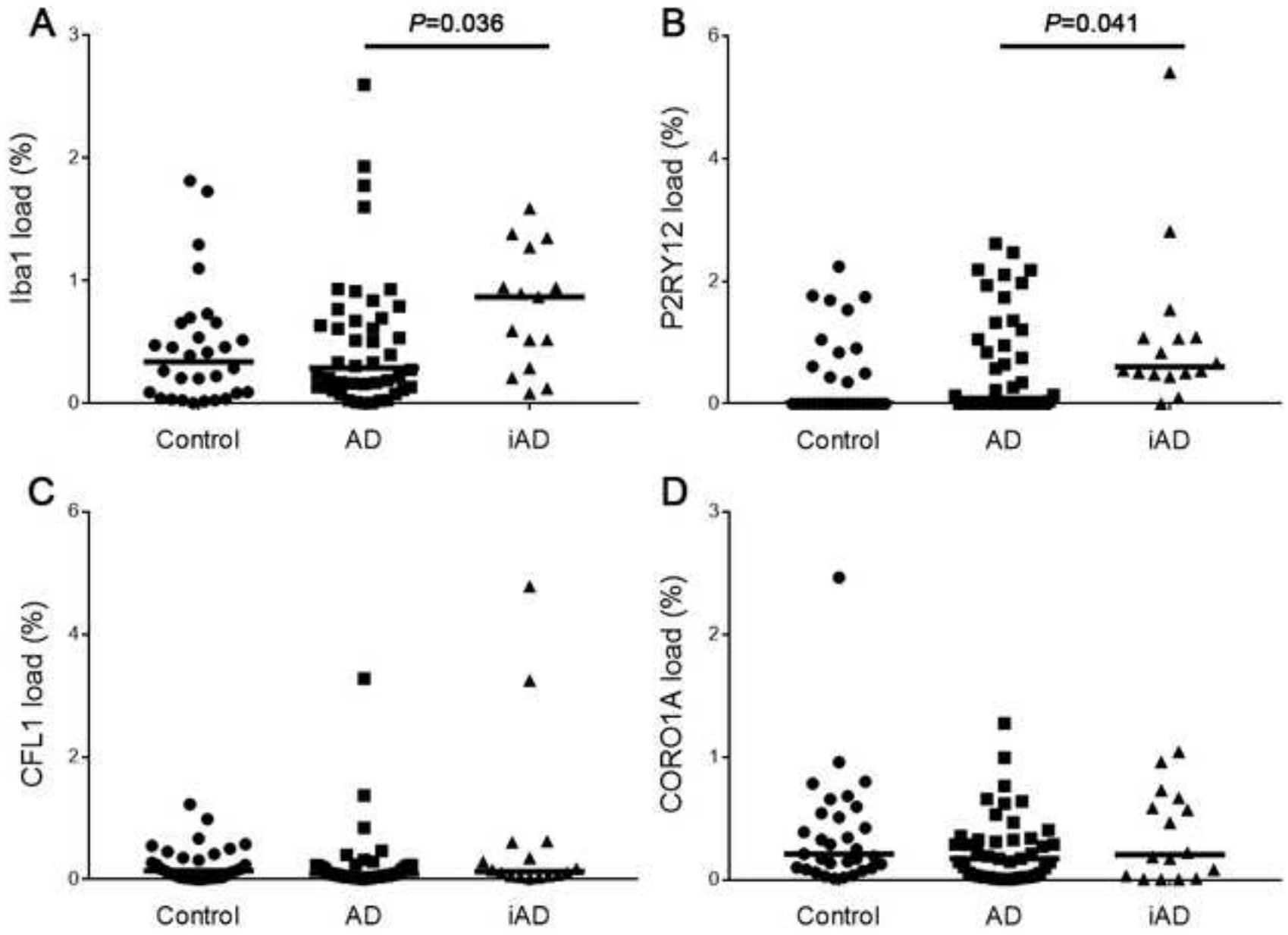


Figure 3

Click here to access/download;Figure;Figure 3.tif



Medicine

UNIVERSITY OF Southampton

7 October 2019

Dear Dr Jeroen Hoozemans,

Revision of the manuscript entitled: Microglial motility in Alzheimer's disease and after Aβ42 immunotherapy: A *post-mortem* study (ANEC-D-19-00388)

We welcome the reviewers' constructive comments and respond to them as below. Our changes are highlighted in the manuscript.

Reviewer #1

I think the question on itself if the motility of microglia was affected by immunization against abeta42 is an interesting one. However, I believe both the methodology as well as text of the study needs major adjustments, which are too extensive for a revision.

We thank the reviewer for their recognition of the importance of the question investigated.

Methodologically

1. Please show a Table 1 with baseline details per case and mention ABC scores, not only Braak stages, if pathology is scored according to Hyman et al.

We are now providing individual baseline details for each case as supplementary Tables 1, 2 and 3. We feel supplementary tables are appropriate due to the number of cases investigated. ABC scores have been provided for all cases when available. However for some control and AD cases, this assessment was not available from the Brain bank. This information has been added in the Material and Methods (page 4).

2. What is the surface size of 1 ROI?

Each ROI had a surface area of 500 x 500 µm (0.25 mm^2). This information has been added in the Material and Methods, section "Quantification of staining" (page 5).

3. Post-mortem delay of a mean of 42 hours is very long! the iAD groups differs substantially in their (also long) post mortem delay of 26 hours, could this have an effect on the Meso Scale Discovery Multiplex Assays?

We previously used this methodology on human *post-mortem* tissue. Cases for this study were selected with the same criteria (*post-mortem* delay less than 72 hours) and provided by

the same brain bank (Rakic et al, Acta Neuropathol Commun 2019). Interestingly, brain protein integrity has been shown to be dependent on the storage temperature, rather than the *post-mortem* delay by the BrainNet Europe Consortium (Ferrer et al, JNEN 2007).
However, to ensure quality of the signal, frozen blocks from 1 multiple sclerosis brain containing chronic inactive, acute or chronic active lesions were used positive controls. This point has been added in the Material and Methods, section “Cytokine assay” (page 6).

4. The authors state the marker P2YR12 does not show clusters, indicated by figure 3. I don't understand the choice for the ptau staining. Why not show a sequential section of an abeta staining? which is more a plaque marker than ptau. Ptau shows neuritic plaques, abeta shows all plaques. In addition, I am not sure if these sections are sequential in the same patient. If they are not, then the images do not underline their statement. Looking closely at the p2yr12 image of figure 3, one might be able to imagine plaque-like clusters in layer 1-2.

Based on the comment from both reviewers, we reviewed all Iba1 and P2RY12 immunolabelled slides and found some P2RY12-positive microglia clusters. This feature was not quantified and thus we decided to remove this sentence as this does not modify the main outcome of the paper.

5. As the authors propose that microglia markers in AD may be correlated with p-tau, I don't understand why they don't show this correlation in their Table 2. If the stainings are performed for ptau, why not scan and correlate them?

We apologise for the confusion and we have now added the correlations performed with ptau in table 2.

6. How can the (i)AD group be Braak stages 5-6, but have ptau levels that are normalized to levels found in healthy controls?

Braak staging is a subjective semi-quantitative assessment of the neuroanatomical distribution and severity of neurofibrillary tangles or cell body ptau pathology, and thus based on the neuroanatomical distribution of ptau-positive neurons rather than the overall amount of ptau present in the cortex (Boche *et al*, Acta Neuropathol 2010). In contrast, our quantitative data presented here relates to a specific neuroanatomical region of cerebral cortex (inferior parietal lobule) in which, in the iAD cases, there is a reduction of Aβ and a concomitant reduction in ptau
To clarify this point, we have now added photos to illustrate pan-Aβ, Aβ42 and ptau in the three groups in Figure 1. We also discussed this point under the section “Aβ and tau pathology“ (page 9) as follows:

“Indeed, our previous studies on the immunised cohort showed that ptau load was reduced after Aβ immunisation, in association with a lower expression of the tau phosphorylating enzyme GSK-3b [2]. Ptau load is mainly constituted of tau-containing dystrophic neurite clusters and ptau in neuronal processes [4], rather than tangles. Nevertheless, despite the decrease in ptau load as assessed by quantification in specific localised neuroanatomical regions of cortex, most of the iAD subjects still had progressed to Braak stages V or VI, as evaluated by the anatomical distribution of neurofibrillary tangles throughout the brain areas. We interpret these findings as indicating that localised removal of Aβ following Aβ immunotherapy results in a corresponding removal of ptau in that location,

consistent with the amyloid cascade hypothesis. However, it does not stop the spread of ptau through the brain, consistent with the evidence for prion-like spread of ptau [4, 33]."

7. Are the findings for the microglia markers IBA1 and P2YR12 in the iAD group correlated with the cases antibody titer in the plasma (Holmes et al., Lancet, 2008)

We performed this statistical analysis and we did not find any significant correlation of Iba1 and P2RY12 load with the antibody titre, as already reported for Iba1 in a previous study (Zotova et al, 2013 Brain). We have reported this analysis in the Discussion (page 9) as follows:

"After Aβ immunotherapy in AD, a 3-fold increase in Iba1 expression and a 6.6-fold increase in P2RY12 expression was observed. Data on anti-Aβ antibody titres measured in the blood during life some years earlier were available [57] and did not correlate with the microglial analyses."

8. The authors state that the immunization against ab42 also worked for other abeta species because of the decrease in the pan-abeta marker. However as the pan-abeta marker entails ab42 as well, this effect can be ab42 driven. The authors need to show an abeta40 or other abeta marker to see if this holds.

We have recently published in the immunised group, reduction of Aβ40 and Aβ43, in addition to Aβ42, having also previously addressed the issue of n-terminus truncation (Nicoll et al. JNEN 2006). This point has now been clarified in the Discussion (page 8):

"This is consistent with our studies on the same immunised cohort using antibodies specific for the Aβ40, Aβ42 and Aβ43 species and reporting significant decrease of the three species (Nicoll et al. JNEN 2006; Jakel et al, Acta Neuropathol Commun 2019)."

<u>Textually</u>

1. The authors critically need to revise the manuscript as some parts are written down in an unconventional manner and some word use or interpunction is not correct.

The grammar and the spelling have been carefully reviewed.

2. In the first paragraph of the discussion, the authors explain about the studies' strengths and weaknesses. This is not the right paragraph for such information. The first paragraph of the discussion should summarize what one did and what the results were.

A short summary of the findings has been added at the start of the Discussion (page 8).

"Our aim was to characterise the expression of motility-related microglial proteins in the human brain and to evaluate its changes in AD and after Aβ immunotherapy. In the present study, we observed increased expression of the microglial homeostatic markers Iba1 and P2RY12 in the immunised group which was associated with a less pro-inflammatory environment than observed in AD."

3. There is inconsistent use of abbreviations. This was the case for ptau, or also abbreviated as (p)tau and sometimes written fully as phosphorylated tau.

This has been corrected throughout the manuscript.

4. Page 7 line 40- 41: AD vs AD cases.

This has been corrected appropriately as iAD *vs* AD.

5. The authors report their p-value inconsistently, sometimes as < ... and sometimes with the exact p-value = 0.045.

Throughout our studies on the immunised cohort, we have consistently reported our p values as recommended by the statisticians working with us: Any p value less than 0.001 has to be reported as "p<0.001"; with the other p values to be reported as the exact value.

Reviewer #2

The strength of this paper is in the description of the autopsy brain sections from patients who had been treated with Abeta immunotherapy (iAD). These specimens are very rare but have potential to greatly enhance understanding of Alzheimer's neuropathology. The results are interesting; however, I feel that analysis could have been more thorough to enable clearer conclusions to be made regarding microglial cell behaviour in these cases.

The following issues should be addressed:

1. The analysis largely depends on quantification of immunolabelling using positive pixel analysis. This seems a very subjective way to determine levels of Iba1, tau etc particularly as the labelling is dependent on readout of an enzymatic reaction (peroxidase) which is a non-linear reaction, followed by imaging and analysis.
None of these procedures appear to have been normalized (there is no explanation of this in the methods). The % area obtained would depend on the level of enzymatic reaction in the batch of slides being processed (it is unclear if all of the slides were processed in parallel?) as well as the exposure of the image in the microscope as well as the post-acquisition image thresholding parameters. A considerable level of variation is expected based on these different parameters. So the significance of the results remains unclear.

We are aware of the non-linearity of the reaction ad thus we are using an image analysis method that quantifies the presence or absence of staining (per pixel) rather than the intensity of the staining, presented as the percentage of the area stained in each image. This method has been widely used by us and others (e.g. Zotova et al, Brain 2013; Paquet et al, J Pathol 2015; Minett et al, J Neuroinflamm 2016).
All our experiments were carefully controlled at each step and included: the use of positive and negative controls, staining of batches containing equal numbers of cases from each group to ensure consistency of the staining between cases. Quantification of the staining was automated using the Olympus slide scanner and macros designed in ImageJ.
These methodological details have been added in the Material and Methods (page 5).

2. Iba-1 positive cell counts could have been done - this would be a more objective read out of microglial cell numbers. How many microglial cells per area? Evidence for proliferation? Likewise, some further analysis of morphology of the cells - degree of ramification etc. Where are the extra Iba1 labelled cells located in the iAD cases? Are they clustered? Are they around Abeta deposits? Or distal? Vascular association?

We agree that these are important questions and we are currently assessing the microglial cell numbers associated with detailed morphological analysis using 3D reconstruction in these cohorts. These findings will result in another publication.

3. Figure 3 images are very pixelated and it is hard to visualise the ptau pathology. It is unclear also which cases these images are derived from? How do the different cases compare? It would be interesting to see the ptau labelling from the control, AD and iAD cases for comparison. Where is the ptau disappearing from in the iAD cases? Are you seeing fewer NFTs? Or is it just that the aggregated tau is being dephosphorylated?

Based on the comment from both reviewers, we reviewed all Iba1 and P2RY12 immunolabelled slides and found some P2RY12-positive microglia clusters. This feature was not quantified and thus we decided to remove this sentence as this does not modify the main outcome of the paper.
In addition, we have added images on figure 1 reflecting the overall amount of pathological markers presents in the three groups.
Additional discussion on ptau reduction has been added in the Discussion (page 9) referring to our previous work on the immunised AD cohort (see response 6 to reviewer 1)

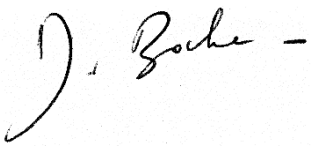
4. It would have been interesting to see the tangle (NFT) counts per area, neuritic plaques per area, the number of neurons per area etc…It would be of interest to show the level of actual neurodegeneration in each group - control, AD and iAD. Does removing the Abeta in the iAD cases also lead to removal of the neuritic component of the plaque?

These findings have been already published by us in several papers with some now cited in the manuscript (e.g. Boche et al. Acta Neuropathologica 2010; Nicoll et al. Brain 2019), consistent with the aim of this study on microglial motility.

5. There is a typo on Results pg 6, line 40-41. "Phosphorylated tau ….decreased in AD vs AD cases (P=0.026)," ..Should read "decreased in iAD vs AD"??

This has been corrected. Thank you

Yours Sincerely



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