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Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly

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ABSTRACT

The pathway leading from amyloid-β deposition to cognitive impairment is believed to be a cornerstone of the pathogenesis of Alzheimer's disease (AD). However, what drives amyloid buildup in sporadic nongenetic cases of AD is still unknown. AD brains feature an inflammatory reaction around amyloid plaques, and a specific subset of the gut microbiota (GMB) may promote brain inflammation. We investigated the possible role of the GMB in AD pathogenesis by studying the association of brain amyloidosis with (1) GMB taxa with pro- and anti-inflammatory activity; and (2) peripheral inflammation in cognitively impaired patients. We measured the stool abundance of selected bacterial GMB taxa (*Escherichia/Shigella*, *Pseudomonas aeruginosa*, *Eubacterium rectale*, *Eubacterium hallii*, *Faecalibacterium prausnitzii*, and *Bacteroides fragilis*) and the blood expression levels of cytokines (pro-inflammatory cytokines: CXCL2, CXCL10, interleukin [IL]-1β, IL-6, IL-18, IL-8, inflammasome complex (NLRP3), tumor necrosis factor-alpha [TNF-α]; anti-inflammatory cytokines: IL-4, IL-10, IL-13) in cognitively impaired patients with (n = 40, Amy+) and with no brain amyloidosis (n = 33, Amy−) and also in a group of

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[1] http://www.centroalzheimer.org/sito/contenuti/ip_lilly_publications/INDIA-FBP_WORKING_GROUP.pdf.

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controls ($n = 10$, no brain amyloidosis and no cognitive impairment). Amy+ patients showed higher levels of pro-inflammatory cytokines (IL-6, CXCL2, NLRP3, and IL-1β) compared with both controls and with Amy− patients. A reduction of the anti-inflammatory cytokine IL-10 was observed in Amy+ versus Amy−. Amy+ showed lower abundance of *E. rectale* and higher abundance of *Escherichia/Shigella* compared with both healthy controls (fold change, FC = −9.6, $p < 0.001$ and FC = +12.8, $p < 0.001$, respectively) and to Amy− (FC = −7.7, $p < 0.001$ and FC = +7.4, $p = 0.003$). A positive correlation was observed between pro-inflammatory cytokines IL-1β, NLRP3, and CXCL2 with abundance of the inflammatory bacteria taxon *Escherichia/Shigella* (rho = 0.60, $p < 0.001$; rho = 0.57, $p < 0.001$; and rho = 0.30, $p = 0.007$, respectively) and a negative correlation with the anti-inflammatory *E. rectale* (rho = −0.48, $p < 0.001$; rho = −0.25, $p = 0.024$; rho = −0.49, $p < 0.001$). Our data indicate that an increase in the abundance of a pro-inflammatory GMB taxon, *Escherichia/Shigella*, and a reduction in the abundance of an anti-inflammatory taxon, *E. rectale*, are possibly associated with a peripheral inflammatory state in patients with cognitive impairment and brain amyloidosis. A possible causal relation between GMB-related inflammation and amyloidosis deserves further investigation.

1. Introduction

Neurodegenerative disorders, including Alzheimer's disease (AD), are characterized by the accumulation of neurotoxic proteins in the brain. In AD, these are amyloid-β (Aβ) and hyperphosphorylated tau, representing the major components of extracellular senile plaques and intracellular neurofibrillary tangles, respectively. The common feature of these proteins is the loss of their physiologic activity and the gain of toxic properties, promoting neurodegeneration. Aβ is widely believed to be the key in AD pathophysiology (Jack et al., 2013). In nongenetic cases of AD, the pathophysiological mechanisms of Aβ deposition and the ensuing neurodegeneration and cognitive symptoms remain to be elucidated, but neuroinflammation seems to play a key role (Heppner et al., 2015). Indeed, in addition to plaques and tangles, AD patients feature central inflammation, mediated by activated microglia, reactive astrocytes, and complement activation, that have been especially observed in the vicinity of amyloid plaques and even in the early stages of AD (Clark and Vissel, 2015; Heneka et al., 2015; Heppner et al., 2015; Latta et al., 2015; Stoeck et al., 2014). Enhanced inflammation occurs also in body fluids of AD patients, such as cerebrospinal fluid and blood (Kauwe et al., 2014; Monson et al., 2014; Nascimento et al., 2014; Vom Berg et al., 2012).

The increased interest in the complex network of inflammatory mediators and the immune system has allowed to identify a growing number of pro-inflammatory molecules involved in central nervous system disorders, such as interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and the inflammasome complex (NLRP3). These have been found associated with cognitive impairment and AD pathology (Chen et al., 2015; Doecke et al., 2012; Leung et al., 2013; Ray et al., 2007; Soares et al., 2012b; Tan et al., 2013). However, the pathophysiological cascade linking inflammation with Aβ deposition is still unknown (Heppner et al., 2015). Some recent observations indicate that a specific subset of the gut microbiota (GMB) can drive neuroinflammation in rodents (Palm et al., 2015; Petra et al., 2015; Erny et al., 2015) and affect brain function and behavior in rodents and humans (Bercik et al., 2011; Diaz Heijtz et al., 2011; Li et al., 2009).

Alterations of GMB composition have been observed in multiple sclerosis (MS) and Parkinson's disease (PD), conditions also featuring neuroinflammation and protein misfolding. Indeed, the removal of GMB in animal models of multiple sclerosis prevents the development of relapsing-remitting demyelination (Berer et al., 2011) and oral ingestion of probiotics attenuates neuroinflammation (Luo et al., 2014; Toumi et al., 2014). In PD, the evidence is even stronger. The deposition of alpha (α)-synuclein, the underlying molecular pathology, has been found both in the digestive tract and enteric nervous system, already in the early phases of the disease (Del Tredici et al., 2010; Goedert et al., 2013; Lebouvier et al., 2010). Moreover, the gut mucosa of Parkinsonian patients shows increased permeability, signs of inflammation and invasion of coliform bacteria (Forsyth et al., 2011), and hosts a peculiar GMB composition, characterized by decreased abundance of *Prevotellaceae* and an increase in *Enterobacteriaceae*, which are also related to the severity of illness (Scheperjans et al., 2015). Importantly, enhanced inflammation, as a consequence of alterations in GMB composition, has been implicated in the initiation of α-synuclein misfolding (Olanow et al., 2014).

To our knowledge, no evidence of GMB alterations has been reported in AD patients yet; however, it has been recently suggested that bacterial endotoxins may play a key role in the inflammatory and pathologic processes associated with amyloidosis and AD (Asti and Gioglio, 2014; Vom Berg et al., 2012), as bacterial components, such as endotoxins, have been found within the typical senile plaque lesions of the AD brain (Asti and Gioglio, 2014; Schwartz, 2013).

The aim of this study was to test, in elder patients with cognitive impairment, the association between brain amyloidosis and (1) candidate GMB taxa with known inflammatory activity (pro-inflammatory: *Escherichia/Shigella* and *Pseudomonas aeruginosa*; anti-inflammatory: *Eubacterium rectale*, *Eubacterium hallii*, *Faecalibacterium prausnitzii*, and *Bacteroides fragilis*) (Bruzzese et al., 2014; Cantarel et al., 2015; De la Fuente et al., 2014; Friedland, 2015); and (2) peripheral inflammation markers implicated in the pathogenesis of AD (pro-inflammatory cytokines: CXCL2, CXCL10, IL-1β, IL-6, IL-18, IL-8, NLRP3, TNF-α; anti-inflammatory cytokines: IL-4, IL-10, IL-13) (Chen et al., 2015; Doecke et al., 2012; Leung et al., 2013; Ray et al., 2007; Soares et al., 2012b; Tan et al., 2013).

2. Methods

2.1. Study design and patients description

The patients have been recruited from a larger study in 18 memory clinics in Eastern Lombardy, Italy, aiming to assess the added value of amyloid imaging in the clinical work-up of patients with cognitive complaints (the Incremental Diagnostic Value of Florbetapir Amyloid Imaging [INDIA-FBP] study) (http://www.centroalzheimer.org/sito/ip_lilly.php). Patients coming to observation with cognitive impairment and AD as a possible etiology were offered, on top and at the end of their routine clinical assessment, amyloid positron-emission tomography (PET) with ^{18}F-Florbetapir. Two hundred and forty-one patients and 26 cognitively healthy elders, mostly patients' spouses, were recruited between August 2013 and December 2014. All patients underwent routine diagnostic work-up as prescribed by their memory clinic specialist,

which in all cases included clinical and neuropsychological assessment. Some patients underwent structural brain magnetic resonance imaging and cerebrospinal fluid analyses for Aβ and total and phosphorylated tau level detection. The Local Ethics Committee at IRCCS San Giovanni di Dio Fatebenefratelli gave ethical approval of the present as an embedded study into INDIA-FBP (authorization no. 57/2014). Accepting patients signed an ad hoc informed consent.

After completion of the INDIA-FBP procedures, 150 patients and controls who were not under antibiotic and anti-inflammatory treatment over the past 3 months or had been diagnosed with major depression or other psychiatric disorders were proposed to contribute samples of stools and blood for the present study. Patients were defined as cognitively impaired in the case they matched these criteria: (1) presence of cognitive complaints reported by patients or proxy or by the doctor; (2) presence of no intracranial metabolic or psychiatric causes of cognitive impairments; (3) presence of abnormal scores in ≥2 cognitive tests; and (4) history of progression of cognitive symptoms. Ten cognitively healthy amyloid-negative controls (HC), 40 cognitively impaired amyloid-positive patients (Amy+), and 33 cognitively impaired amyloid-negative patients (Amy−) gave their consent to participate to the study and donate blood and stool samples.

Patients underwent clinical assessment including medical cognitive and functional history, physical examination including collection of height and weight, neurological examination, drug history, mood and behavior assessment, and neuropsychological assessment including the Mini-Mental State Examination (MMSE) (Folstein et al., 1975). Body mass index (BMI) was defined as weight/height^2 and measured in kg/cm^2. The neuropsychological battery consisted of tests tapping verbal and nonverbal learning, immediate memory, abstract thinking, visuospatial planning, constructional apraxia, verbal fluency, and comprehension. Medial temporal atrophy and subcortical cerebrovascular disease were assessed with validated and largely used visual rating tools (the medial temporal atrophy [Scheltens et al., 1993]) and the age-related white matter change scales (Wahlund et al., 2001), ranging from 0 to 4 and 0 to 30, respectively, where 0 means no abnormal changes. Neuropsychological test scores have been reported in Supplementary Table 1 and current medication in Supplementary Table 2.

2.2. Amyloid PET

Patients underwent amyloid PET at the Nuclear Medicine Service of Spedali Civili and Fondazione Poliambulanza in Brescia with GE Discovery 690 and Siemens Biograph 40m PET–computed tomography scanners, respectively. PET was a 10-minute (two 5-minute frames) 3-dimensional acquisition, 50 minutes after the injection of an intravenous bolus of 370 MBq (10 mCi) of ^{18}F-Florbetapir (Clark et al., 2012). Attenuation correction was calculated based on the co-acquired computed tomography. PET images were reconstructed onto a 128 × 128 matrix with slice thickness of 3–3.3 mm, using a 2- to 3-mm Gaussian post-reconstruction filter. Subjects were categorized into Amy+ and Amy− after a validated procedure (http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM240266.pdf). PET exemplars from an HC, an Amy+, and an Amy− participant can be found in the Supplementary Material (see Supplementary Fig. 1).

Amyloid positivity was visually rated independently by 2 nuclear physicians and blind to patients' clinical information. A third expert was in charge of adjudicating discordant cases, which amounted to 12% of the total.

2.3. Stool sample collection and DNA isolation

Stool samples were collected by participants at their own home in a sterile plastic cup, stored at −20 °C, and delivered to IRCCS Fatebenefratelli Institute within the following 24 hours, where they were stored at −20 °C until processing. Microbial DNA was extracted 3 times in 3 different sections from each patient's stool taking 200 mg of stool per time, using the QIAamp DNA Stool Mini Kit (Qiagen) and according to the manufacturer's instructions, with the additional glass-bead beating steps on a Mini-beadbeater (FastPrep; Thermo Electron Corp). DNA samples coming from the same patients were subsequently tested as technical triplicates. DNA was quantified using a NanoDrop ND-1000 spectrophotometer, and DNA integrity and size were assessed by 1.0% agarose gel electrophoresis on gels containing 0.5 mg/mL ethidium bromide. DNA samples were then stored at −20 °C until subsequent analyses.

2.4. Bacterial DNA quantification in stools

The abundance analyses of the selected bacterial taxa (*Escherichia/Shigella*, *P. aeruginosa*, *E. rectale*, *E. hallii*, *F. prausnitzii*, and *B. fragilis*) were carried out using the Microbial DNA qPCR Assay Kit (Qiagen, Crawley, UK) and a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Fifty nanograms of total DNA was used for each sample. Pan Bacteria primers designed to detect the broadest possible collection of bacteria hosted in the human gut were measured together with candidate taxa to normalize the abundance of each candidate bacterial taxon.

The kits for the detection of the previously mentioned taxa do not provide the primer sequences, and we here include the code number for each assay (Pan Bacteria 1: BPCL00360AR; *Escherichia/Shigella*: BPID00146AR; *P. aeruginosa*: BPID00288AR; *E. rectale*: BPID00149AR; *E. hallii*: BPID00147AR; *F. prausnitzii*: BPID00154AR; and *B. fragilis*: BPID00146AR).

The abundance of each taxon was then calculated according to the comparative Ct method (−ΔΔCt method) (Schmittgen and Livak, 2008) and following the Microbial DNA qPCR Assay Kit protocol (https://www.qiagen.com/us/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/microbial-dna-qpcr-assay-kits/) where the control subjects (HC) have been used as reference group. When comparing groups, this method allows to obtain a fold change (FC) value of differences for each candidate bacteria taxon.

2.5. Gene expression analyses of inflammatory molecules in blood

Isolation of total RNA was performed using the PAXgene blood miRNA kit, according to the manufacturer's recommended protocol (Qiagen). RNA quantity and quality were assessed by evaluation of the A260/280 and A260/230 ratios, using a Nanodrop spectrophotometer (NanoDrop Technologies, USA), and RNA samples were then kept at −80 °C until their processing for gene expression analyses. Gene expression levels were analyzed by a 384-well qRT-PCR instrument (Bio-Rad Instrument), using the iScriptTM 1-step RT-PCR kit for probes (Bio-Rad Laboratories) and Applied Bio-System Assays (Gene Expression Assays: *CXCL2, CXCL10, IL-1β, IL-6, IL-18, IL-8, NLRP3, TNF-α, IL-4, IL-10, IL-13*) as previously reported (Cattaneo et al., 2013). Samples were run in triplicates, and each target gene was normalized to the expression of 3 housekeeping (HK) genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-2-microglobulin (*B2M*), and β-actin. All the assays for the gene expression analyses of both target and HK genes were purchased from Life Technologies (Monza, Italy). For each sample, 50 ng of RNA were added to the Real-Time PCR Mix. Thermal cycling was initiated

with an incubation at 50 °C for 10 minutes, followed by 5 minutes at 95 °C. After this initial step, 39 cycles of polymerase chain reaction (PCR) were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 seconds to enable the melting process and then for 30 seconds at 60 °C for the annealing and extension reactions. The expression of target genes was calculated according to the Ct method (−ΔΔCt method) (Schmittgen and Livak, 2008), where the control subjects (HC) have been used as a reference group. We thus obtained, as output, a relative expression ratio (R) value for each cytokine in each sample.

2.6. Statistical analysis

Parametric (analysis of variance, t-test and chi-square) and nonparametric (Mann–Whitney) tests were applied to compare dichotomous and continuous variables (demographic and clinical features) between the study groups or other variables. Pearson linear correlation (r) and Spearman rank correlation (rho) were used for Gaussian and non-Gaussian distributed variables respectively, to evaluate correlations between demographic and clinical variables. For the evaluation of correlations between stool bacteria and blood inflammation biomarkers, the algorithm for smoothing scatterplots by robust locally weighted regression (Cleveland et al., 1992) was applied to draw the fitted curves.

We chose to test MMSE, BMI, age, and gender as possible confounders based on literature evidence and checked empirically their confounder status, that is, the association with both outcome and treatment/exposure. Moreover, we have also tested the association between cholinergic drugs and bacteria taxa, based on the notion that these drugs exert an effect on intestinal motility.

Finally, a model-based evaluation of confounders was carried out in terms of goodness-of-fit indexes (Akaike Information Criterion and Bayesian Information Criterion indexes for generalized linear model) and "parsimony" criterion other than "change-in-estimate evaluation."

The normality assumption of cytokine blood levels and bacteria taxa were evaluated by Tukey boxplot (with 1.5 interquartile range) and Quantile-Quantile plot inspection and tested by Shapiro–Wilk and Kolmogorov–Smirnov tests. According to the distribution of dependent variables (bacteria taxa abundances or cytokine messenger RNA levels), we then applied (1) generalized linear models (GLM), with log-link function for the gamma-distributed data, for the evaluation of all bacteria taxa across groups; and (2) analysis of covariance models for cytokine blood levels. GLM and analysis of covariance were adjusted for MMSE, BMI, age, and gender according to the results of the correlation/association analysis.

Post hoc group comparisons were evaluated by Bonferroni adjustment. Statistical significance was set at $p < 0.05$. Statistical analyses were performed by SPSS, version 22.0, and R: A language and environment for statistical computing, version 3.2.5, R Foundation for Statistical Computing, Vienna, Austria.

3. Results

3.1. Clinical sample description

The 3 groups were similar for age, gender, and BMI. Amy+ patients had lower cognitive performances than both Amy− and HC (Table 1). Both neurodegeneration in the medial temporal lobe and microvascular white matter changes were similar in Amy+ and Amy−. Indeed, Amy− and Amy+ patients did not show neither significant difference on medial temporal atrophy (Scheltens scale: mean ± standard deviation 1.8 ± 1.1 and 2.0 ± 0.8, $p = 0.670$) nor white matter changes (age-related white matter change scale: mean ± standard deviation 2.5 ± 3.1 and 1.8 ± 2.2, $p = 0.520$).

Table 1
Demographic and clinical features of study participants

Feature	Amy+ ($n = 40$)	Amy− ($n = 33$)	HC ($n = 10$)	Significance (p)
Age (y)	71 ± 7	70 ± 7	68 ± 8	n.s.
Females	20/40	18/34	6/10	n.s.
BMI	24.5 ± 3.5	25.6 ± 3.7	24.3 ± 2.9	n.s.
MMSE	21.3 ± 6.1	25.5 ± 3.9	28.3 ± 1.1	$p < 0.01$ Amy+ vs Amy−; $p < 0.05$ Amy+ vs HC

Numbers denote mean ± standard deviation; p indicates the significance of the group differences on Student t or chi-square test.
Key: Amy+, cognitively impaired patients with brain amyloidosis; Amy−, cognitively impaired patients with no brain amyloidosis; BMI, body mass index; HC, patients with no brain amyloidosis and no cognitive impairment; MMSE, Mini-Mental State Examination; n.s., not significant.

3.2. Candidate bacteria taxon abundance in the stools

First, we assessed the association between abundance of all bacteria taxa with demographic and clinical (MMSE, BMI, age gender, and assumption of cholinergic drug) variables. We found a significant association only between *P. aeruginosa* and BMI (rho = 0.41, $p = 0.037$). Thus, BMI was included, together with the MMSE, as a covariate in the GLM with *P. aeruginosa* as dependent variable. No correlations with age (p values of Spearman correlation >0.097) and no associations (evaluated by Mann–Whitney tests) with gender ($p > 0.100$) were found for all bacteria taxa. The assumption of cholinergic drugs was differentially distributed between Amy+ and Amy− patients (chi-squared $p < 0.001$), but the association with bacteria taxa abundances was not significant ($p > 0.050$) for all bacteria taxa.

The distribution of all bacteria taxa showed a significant density mass close to 0 and a continuous, right-skewed distribution elsewhere indicating a gamma distribution (p values of Kolmogorov–Smirnov test for gamma distributions >0.16 for all bacteria taxa). We thus applied GLM models to all bacteria taxa. In keeping with the results of the correlation/association analysis, we adjusted all analyses for MMSE, except *P. aeruginosa* analysis that was adjusted also for BMI.

In Amy+, we found a specific pattern of alterations in bacteria taxa abundance compared with HC and to Amy+. In particular, Amy+ showed lower abundance of *E. rectale* and higher abundance of *Escherichia/Shigella* compared with both HC (FC = −9.6, $p < 0.001$ and FC = +12.8, $p < 0.001$; MMSE $p = 0.029$ and 0.104, respectively) and to Amy− (FC = −7.7, $p < 0.001$ and FC = +7.4, $p = 0.003$; MMSE $p = 0.053$ and 0.205, respectively). Moreover, Amy+ showed lower abundance of *B. fragilis* than HC (FC = −24.5, $p = 0.032$; MMSE $p = 0.369$). No difference in the abundance of the other bacteria taxa was observed (Fig. 1).

3.3. Expression of inflammation biomarkers in the blood

The distribution of gene expression values was Gaussian for all cytokines (p-values of Kolmogorov–Smirnov test for normal distributions always >0.100). We assessed possible associations between the demographic variables age, gender, and BMI, with the levels of the cytokines, and we found a significant correlation between NLRP3 and age ($r = 0.27$, $p = 0.013$), between IL-6 and age ($r = -0.24$, $p = 0.032$), and between IL-18 and gender ($r = 0.25$, $p = 0.022$). Thus, in addition to MMSE, we have also included age and/or gender as covariates, whenever necessary, in the analyses.

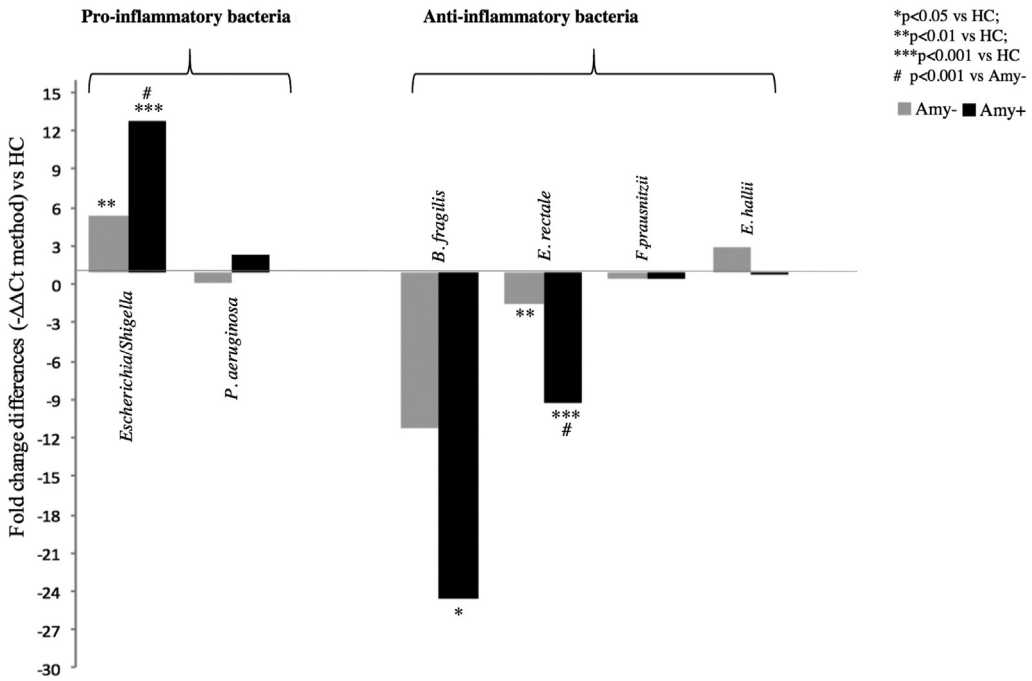

Fig. 1. Abundance of bacterial taxa in the stools of study participants. Bars denote fold changes (FCs) of difference in amyloid-positive (Amy+) and amyloid-negative (Amy−) patients versus control subjects (HC). The FC has been calculated using control subjects as reference (represented by the threshold line at 0), according to the ΔΔCt method (for details, see Section 2). Statistical significance is represented by * at $p < 0.05$, ** at $p < 0.01$, and *** at $p < 0.001$ comparing Amy+ and Amy− versus HC and statistical significance by # at $p < 0.001$ comparing Amy+ versus Amy−.

As shown in Fig. 2, Amy+ showed a specific pattern of higher levels of 4 pro-inflammatory cytokines (NLRP3, CXCL2, IL-6, and IL-1β), as their levels were different compared with both HC and Amy−. In particular, we found significantly increased expression of NLRP3, CXCL2, IL-6, and IL-1β in Amy+ versus HC (+22%, $p = 0.030$; +36%, $p < 0.001$; +22%, $p = 0.030$; and +40%, $p = 0.004$, respectively) and versus Amy− (+19%, $p = 0.006$; +24%, $p < 0.001$; +32%, $p < 0.001$; and +22%, $p = 0.040$).

Amy+ and Amy− showed a similar pattern of increased expression of TNF-α compared with HC (+29%, $p < 0.001$ and +31%, $p < 0.001$, respectively). With regard to anti-inflammatory cytokines, Amy+ patients showed a significantly reduced expression of IL-10 but only compared with Amy− (−25%, $p = 0.007$); no significant difference in the expression levels of CXCL10, IL-18, IL-8, IL-4, and IL-13 was observed across groups (all $p > 0.05$).

3.4. Correlation between stool bacteria and blood inflammation biomarkers

We performed Spearman correlation analyses between cytokines and bacteria strains focusing on cytokines and strains whose pattern was significantly different in Amy+ patients both versus HC and Amy−.

In particular, we correlated the blood levels of the cytokines CXCL2, IL-6, NLRP3, IL-1β, and IL-10 with the stool abundance of *Escherichia/Shigella* and *E. rectale*. We found a positive correlation among the pro-inflammatory cytokines IL-1β, NLRP3, and CXCL2 with abundance of *Escherichia/Shigella* (rho = 0.60, $p < 0.001$; rho = 0.57, $p < 0.001$; rho = 0.30, $p = 0.007$, respectively) (Fig. 3). A negative correlation was observed among blood levels of the pro-inflammatory cytokines IL-1β, NLRP3, and CXCL2 with stool abundance of anti-inflammatory bacteria *E. rectale* (rho = −0.48, $p < 0.001$; rho = −0.25, $p = 0.024$; rho = −0.49, $p < 0.001$, respectively) and a positive correlation between IL-10 blood levels and *E. rectale* (rho = 0.30, $p = 0.030$) (Fig. 4).

Most of the correlations hold significant also when Amy+ and Amy− were investigated separately. In particular, we found a positive correlation between the blood levels of IL-1β and NLRP3 with *Escherichia/Shigella* in both Amy+ (rho = 0.34, $p = 0.032$ and rho = 0.65, $p < 0.001$, respectively) and Amy− (rho = 0.45, $p = 0.009$ and rho = 0.47, $p = 0.007$) and a negative correlation between the levels of IL-1β and CXCL2 with *E. rectale* in Amy+ (rho = −0.37, $p = 0.020$ and rho = −0.32, $p = 0.040$) and Amy− (rho = −0.43, $p = 0.014$ and rho = −0.37, $p = 0.040$).

Importantly, when we evaluated the MMSE contribution in these analyses (through GLM with MMSE as covariate), we found no influence on the main effect for all tested correlations (all $p > 0.05$).

4. Discussion

In the present study, we have investigated the association of brain amyloidosis with candidate GMB taxa, known to have inflammatory properties, and peripheral blood inflammation biomarkers. We found that subjects with cognitive impairment and brain amyloidosis had lower abundance of the anti-inflammatory *E. rectale* and higher abundance of the pro-inflammatory *Escherichia/Shigella* in their stools when they were compared to both a group of control subjects and also to a group of subjects with cognitive impairment and amyloid negative.

Consistently, Amy+ showed higher levels of the pro-inflammatory cytokines IL-6, CXCL2, NLRP3, and IL-1β and reduced levels of the anti-inflammatory cytokine IL-10. Interestingly, the abundance of *Escherichia/Shigella* correlated positively with the levels of IL-1β, CXCL2, and NLRP3, whereas *E. rectale* correlated negatively with the levels of IL-1β, CXCL2, and NLRP3 and positively with IL-10.

To our best knowledge, this is the first study reporting clinical evidence of GMB alterations in patients with brain amyloidosis. Previous experimental and neuropathologic studies have suggested a possible involvement of GMB composition in AD

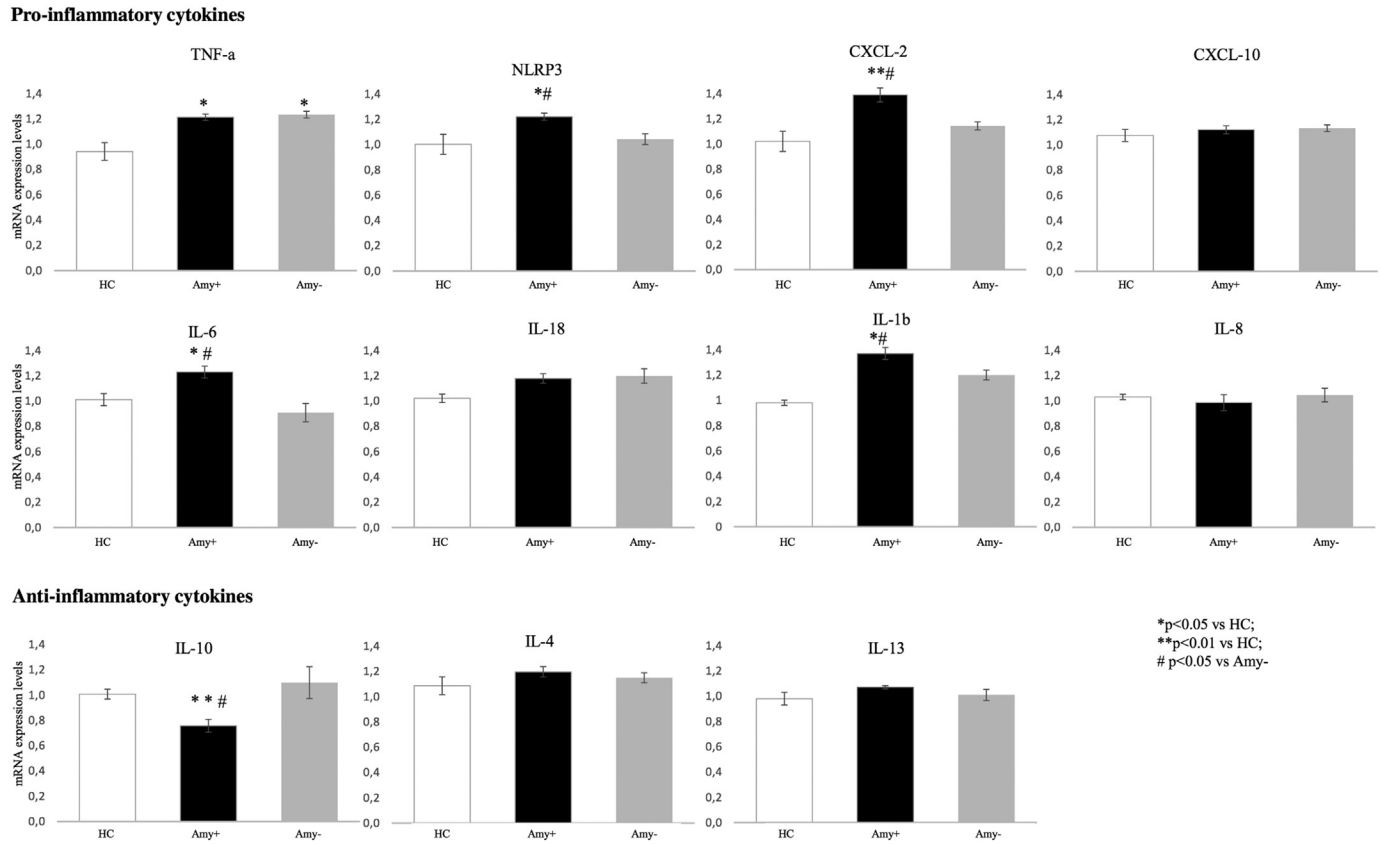

Fig. 2. Expression levels of inflammation-related cytokines in the blood of study participants. Data are shown as relative expression ratio of gene expression in control subjects (HC) and amyloid-positive (Amy+) and amyloid-negative (Amy−) patients. Bars denote mean ± standard error. Statistical significance is represented by * at $p < 0.05$ and ** at $p < 0.001$ comparing Amy+ or Amy− versus HC and statistical significance by # at $p < 0.05$ comparing Amy+ versus Amy−.

pathogenesis. Recent data show that bacterial endotoxins may exert a key role in the inflammatory and pathologic processes associated with amyloidosis and AD (Asti and Gioglio, 2014), as a co-incubation of Aβ with *Escherichia* endotoxins caused a potentiation of the in vitro Aβ fibrillogenesis. Bacterial components, such as endotoxins, have indeed been found within the typical senile plaque lesions of the AD brains (Asti and Gioglio, 2014; Schwartz, 2013). Recently, Kamer et al. (2016) have shown that clinical measures of periodontal disease in cognitively normal healthy elders are positively associated with the severity of brain amyloid accumulation assessed by [^{11}C] PIB-PET, suggesting that dysbiosis related to chronic periodontal inflammation/infection may be involved in AD pathogenesis.

In our study, the abundance of the genus *Escherichia/Shigella* was significantly increased in Amy+ compared with Amy− patients. *Escherichia/Shigella* has been associated with a pro-inflammatory status (Morgan, 2013; Soares et al., 2012a) and, in a recent study, Small et al. (2013) found that persistent infection with adherent and

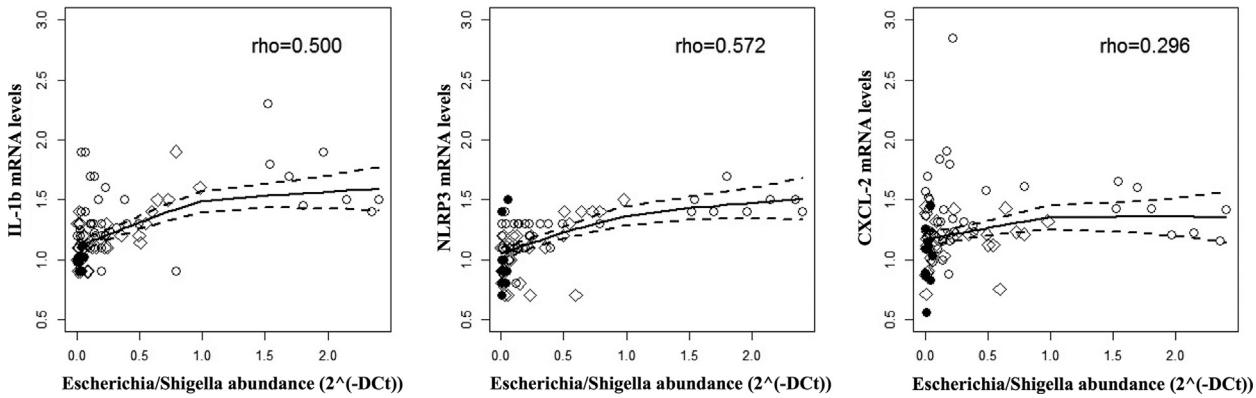

Fig. 3. Spearman correlation of cytokines blood levels with *Escherichia/Shigella* stool abundance in study participants. Cytokines (in the y axis) and *Escherichia/Shigella* (in the x axis) were selected based on the significance of the difference between groups. Full dots denote control subjects and open dots denote amyloid-positive (Amy+), and open diamonds amyloid-negative patients (Amy−). The graph shows Spearman correlations rho values and locally weighted polynomial fitted curves (in black line) with relative 95% confidence band (in dotted line) obtained by a smoother span parameter equal to 100%.

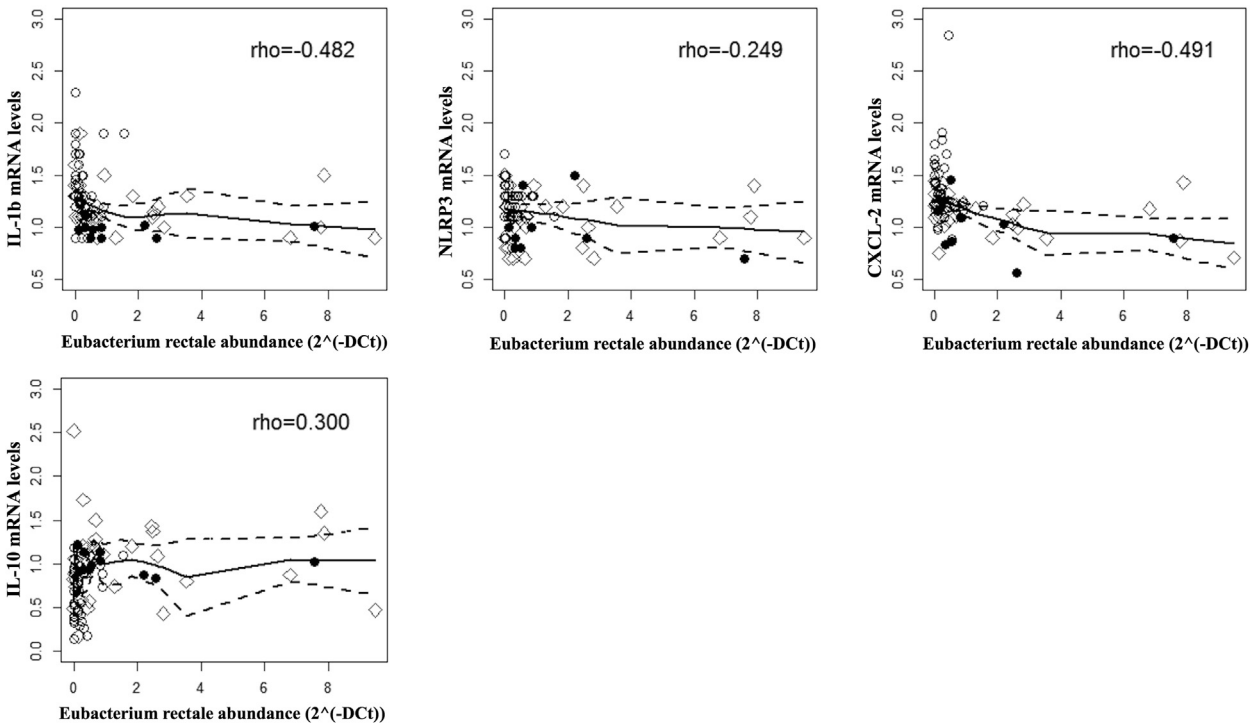

Fig. 4. Spearman correlation of cytokines blood levels with *Eubacterium rectale* abundance in the stools in study participants. Cytokines (in the y axis) and *E. rectale* abundance (in the x axis) were selected based on significance of the difference between groups. Full dots denote control subjects and open dots denote amyloid-positive (Amy+), and open diamonds amyloid-negative patients (Amy−). The graph shows Spearman correlations rho values and locally weighted polynomial fitted curves (in black line) with relative 95% confidence band (in dotted line) obtained by a smoother span parameter equal to 100%.

invasive *Escherichia* led to chronic and persistent peripheral inflammation. Also, De la Fuente et al. (2014) reported an ability of the genus *Escherichia* to induce the production of pro-inflammatory cytokines through NLRP3-dependent mechanism. Interestingly, in line with the studies mentioned previously, we found a positive correlation between changes in the abundance of *Escherichia/Shigella* and changes in the levels of the pro-inflammatory molecules IL-6, CXCL2, and NLRP3, which is consistent with a possible cause–effect relationship (Huang et al., 2015). Of particular interest is the NLRP3 inflammasome, whose activation leads to the induction of inflammatory processes, including the maturation and the release of several pro-inflammatory cytokines and chemokines. Once activated, it can also promote the formation of inflammatory crystals and protein aggregates, including Aβ. NLRP3 production has been found enhanced in the brain of AD patients (Halle et al., 2008; Martinon et al., 2009), and an NLRP3 inflammasome deficiency resulted in decreased pro-inflammatory cytokines release and decreased deposition of Aβ in the APP/PS1 animal model of AD (Heneka et al., 2013).

We also identified a significant reduction in *E. rectale* abundance in Amy+ compared with Amy− subjects. *E. rectale* is a bacteria known to produce butyrate (an anti-inflammatory compound) that plays key protective roles against inflammation (Pryde et al., 2002). A reduction in its abundance correlated negatively with pro-inflammatory molecules in our sample, denoting enhanced sensitivity to inflammatory processes. This is in line with recent evidence indicating that an increase in *E. rectale* abundance is associated with lesser degree of inflammation. An increase in *E. rectale* also predicted positive response to treatment with anti-TNF-α, in patients with inflammatory bowel disease (Kolho et al., 2015).

Our findings may also be interpreted at the light of recent data regarding other neurological disorders, including MS and PD that share with AD neuroinflammation and protein misfolding, respectively. In MS, studies in animal models and in germ-free mice (Forsythe and Kunze, 2013; Lee et al., 2011) showed that GMB modifications may cause the activation of immune and inflammatory responses that can extend beyond the gut, up to the brain. In PD patients, changes in the GMB composition have been recently observed (Hasegawa et al., 2015; Keshavarzian et al., 2015; Scheperjans et al., 2015) and also as alterations in the gut barrier function, membrane permeability, and inflammatory mediators production have been reported as affecting not only gut immune epithelial cells and immune system cells but also neurons and glial cells in PD patients (Forsyth et al., 2011). Moreover, specific bacteria taxa or their metabolites may trigger α-synuclein misfolding (Chorell et al., 2015; Evans et al., 2015).

Two interesting studies (Hasegawa et al., 2015; Keshavarzian et al., 2015) provide evidence that pro-inflammatory dysbiosis could trigger inflammation-induced misfolding of α-synuclein and promote the development of PD. In the study of Keshavarzian et al. (2015), mucosal-associated and microbiota compositions were analyzed using high-throughput ribosomal RNA gene sequencing, showing higher abundance of the anti-inflammatory butyrate-producing bacteria in the feces of controls compared with PD patients. Moreover, an increase of *Faecalibacterium* and a reduction of *Proteobacteria* were found in the mucosa of controls compared with PD patients. In the other study, Hasegawa et al. (2015) measured serum markers and quantified 19 fecal bacterial taxa by quantitative PCR in a group of PD patients compared with healthy controls. The authors found that the abundance of *Lactobacilli* was higher, whereas those of *Clostridium coccoides* and *B. fragilis* was lower in PD patients than in controls.

In this scenario, we can hypothesize that the role of GMB composition in AD may be multiple. Indeed, specific GMB taxa may cause, as also supported by our data, the induction of immune and inflammatory responses in the brain, which in turn may induce Aβ

deposition. However, we cannot exclude that alternative mechanisms exist, including the possible ability of GMB taxa or their metabolites to directly trigger protein misfolding and aggregation. Toxic forms of neurodegenerative diseases have been described, such as amyotrophic lateral sclerosis–dementia complex of Guam because of *N*-β-methylamino-L-alanine produced by *Cyanobacteria* (Cox et al., 2005).

This study has some limitations. First, the cross-sectional nature of the study prevents to test a possible causal relationship and any pathophysiological pathway leading from GMB composition, to neuroinflammation, to brain amyloidosis, and lately to AD. Second, we have not used the most current standard methods for GMB assessment that is the next-generation sequencing, but we have investigated 6 specific taxa. However, if the added value of next-generation sequencing is to identify, by using a hypothesis free approach, all the possible taxa differentially modulated in a pathologic condition, it is also true that for most of bacterial taxa the physiological properties are still poorly understood. This is the reason we used specific hypothesis driven approach and thus we selected taxa with inflammatory properties as we aimed to assess the specific hypothesis that alterations in the abundance of specific gut taxa may be associated with alterations in the inflammatory status in the periphery and amyloidosis at the central level.

Third, we lack information about the dietary habits of our patients. We are aware that long-term dietary differences can have major effects on the microbiome composition (Cryan and Dinan, 2012; Holmes et al., 2012), and this issue deserves further investigation. The 3 points difference in the MMSE of Amy+ and Amy− subjects may be interpreted as a downstream detrimental effect of brain amyloidosis on cognitive performance. However, this deserves further investigation, both as to its possible significance as to the role of GMB, and for careful control of this confounder in future studies.

In conclusion, our data indicate that an increase in the abundance of a pro-inflammatory GMB taxon, *Escherichia/Shigella*, and a reduction in the abundance of an anti-inflammatory taxon, *E. rectale*, are possibly associated with a peripheral inflammatory state in patients with cognitive impairment and brain amyloidosis. This finding leads to the hypothesis that the GMB composition may drive peripheral inflammation, contributing to brain amyloidosis and, possibly, neurodegeneration and cognitive symptoms in AD. Further studies are needed to explore this possible causative role of GMB composition in inflammatory changes and brain amyloidosis.

Disclosure statement

Giovanni Frisoni has served in advisory boards for Roche, Lilly, BMS, Bayer, Lundbeck, Elan, Astra Zeneca, Pfizer, Taurx, Wyeth, GE, and Baxter. He received research grants from Wyeth International, Lilly International, Lundbeck Italia, GE International, Avid/Lilly, Roche, Piramal, and the Alzheimer's Association. In the last 2 years, he received speaker honoraria from Lundbeck, Piramal, GE, and Avid/Lilly; Marina Boccardi received a research grant from Piramal; Alessandro Padovani received honoraria for speaking at symposia from General Electrics, Lundbeck, and Novartis; in addition, he received honoraria for participating at Scientific Advisory Board from General Electrics, Eli-Lilly, and Novartis. The other authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2016.08.019.

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