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

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Keywords: cognitive impairment, brain amyloidosis, inflammation, gut microbiota, neurodegeneration

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Manuscript Region of Origin: ITALY

Abstract: The pathway leading from beta-amyloid deposition to cognitive impairment is believed to be a cornerstone of the pathogenesis of Alzheimer's disease (AD). However, what drives amyloid build-up in sporadic non-genetic 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 (i) GMB taxa with pro- and anti-inflammatory activity, and (ii) 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, IL-1β, IL-6, IL-18, IL-8, NLRP3, 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 controls (n=10, no brain amyloidosis and no cognitive impairment, HC).
Amy+ patients showed higher levels of pro-inflammatory cytokines (IL-6, CXCL2, NLRP3 and IL-1β) compared to both controls and to Amy- patients. A reduction of the anti-inflammatory cytokine IL-10 was observed in Amy+ versus Amy-. Amy+ showed lower abundance of Eubacterium rectale and

higher abundance of Escherichia/Shigella as compared to both HC (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 Eubacterium 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, Eubacterium 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.

*Verification

## Potential Conflicts of Interest

Giovanni Frisoni has served in advisory boards for Roche, Lilly, BMS, Bayer, Lundbeck, Elan, Astra Zeneca, Pfizer, Taurx, Wyeth, GE, Baxter. He received research grants from Wyeth Int.l, Lilly Int.l, Lundbeck Italia, GE Int.l, Avid/Lilly, Roche, Piramal, and the Alzheimer's Association. In the last two years he received speaker honoraria from Lundbeck, Piramal, GE, Avid/Lilly;

Marina Boccardi received a research grant from Piramal;

Alessandro Padovani received honoraria for speaking at symposia from General Electrics, Lundbeck, Novartis; in addition, he received honoraria for participating at Scientific Advisory Board from General Electrics, Eli-Lilly and Novartis.

The other authors have not conflicts of interest to declare.

## Highlights

- We aimed to investigate the possible role of the GMB in AD pathogenesis
- GMB taxa were measured in the stools and inflammatory cytokines in the blood of Amy-, Amy+ cognitively impaired patients and controls;
- Amy+ patients have higher abundance of pro-inflammatory GMB taxa and higher peripheral inflammation
- pro-inflammatory GMB composition is associated with peripheral inflammation in patients with cognitive impairment and brain amyloidosis

***Rebuttal Letter**

Provincia Lombardo - Veneta
Ordine Ospedaliero di S. Giovanni di Dio – Fatebenefratelli

**"CENTRO S. GIOVANNI DI DIO - FATEBENEFRATELLI"**
*Istituto di Ricovero e Cura a Carattere Scientifico*
(D.I. 19 Dicembre 1996)

Brescia, August 23rd, 2016

Dear Prof Rapp,

We thank you for the possibility to improve our manuscript by addressing the comments of reviewer 2. We are particularly encouraged by the positive appraisal of the other two reviewers who have not commented further. The latest revisions have been highlighted in yellow for your convenience.

Please find attached revision 2 of our manuscript entitled "*Association of brain amyloidosis with pro-inflammatory gut bacterial strains and peripheral inflammation markers in cognitively impaired elderly*" that we re-submit as a ***Research Article*** to Neurobiology of Aging.

We hope that you will find this version suitable for publication in your journal.

Yours sincerely,

Annamaria Cattaneo, on behalf of all authors

# Reviewers' comments to revision 1

## Reviewer #2

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confounders here?

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More recent literature (see e.g. *van Belle et al., 2004, Biostatistics, Chap. 11, Wiley Series in Probability and Statistics;* and *Rosner B., 2011, Fundamentals of Biostatistics, Chap. 13*) suggests that substantive considerations, based on clinical and/or biological rather than purely statistical evidence, should drive the choice of confounders. Accordingly, we based our choice of confounders on literature evidence, and checked empirically their confounder status, i.e. the association with both outcome and treatment/exposure. Finally, a model-based evaluation of confounders was carried out in terms of goodness of fit indexes (AIC and BIC index for Generalized Linear Model) and "parsimony" criterion other than change-in-estimate evaluation.

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Nevertheless, the p-value of each covariate has now been reported in the Result Section "Candidate bacteria taxon abundance in the stools".

- In the results, the authors show only fold-change or percent change values. However, also the absolute values for abundance (pan bacterial primers were apparently also used) and cytokine levels should be presented for each study group, eg in the supplement.

In Real Time PCR, the most common and used quantification method is the *relative quantification approach* where target genes are normalized to housekeeping or reference genes. This method does not allow to compute absolute values. The values of each target taxon or cytokine have thus been normalized to the values of pan bacteria taxa or housekeeping genes respectively. We have already used this commonly and widely used method in cytokine gene expression analyses (Hepgul et al., 2016 *Neuropsychopharmacology*; Cattaneo et al., 2013 *Int J of neuropsychopharmacology*; Di Nicola et al., 2013, *Brain Behav Immun*; Anacker et al. 2013, *PNAS*).

• In the previous version the authors stated that subjects also underwent a detailed assessment of concomitant medication without showing results and accounting for it in the analysis. Now, the authors do not mention any medication assessment anymore. Is the data available or not? Cognitively impaired patients are frequently treated with cholinergic drugs that frequently have gastrointestinal side effects and could possibly alter microbiota. So, if available, this must be assessed and adjusted if necessary. Otherwise, it must be stated that medication effects cannot be ruled out in this study.

We thank the reviewer for this comment. In the manuscript, on page 8, we mentioned that "*Patients underwent clinical assessment including medical cognitive and functional history, physical examination including collection of height and weight, neurological examination, drug history, mood and behaviour assessment, and neuropsychological assessment including the Mini Mental State Exam (MMSE)*". In the current version we have added a table (Supplementary Table 2) listing all concomitant medications.

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• On page 12, the authors report a significant correlation between IL-18 and gender. Gender is a categorical variable so probably another test should be used.

Indeed, we inappropriately used the term "correlation". In line with what reported in the Statistical Analysis section "*…parametric tests (ANOVA, t-test and Chi-square) were applied to compare dichotomous and continuous variables (demographic and clinical features)",* we used t-test to assess the association between IL-8 and gender. We have now changed "correlation" into "association" in the pertinent passage.

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As mentioned above, bacterial abundances are skewed variables and they have outliers; for this reason, we have adopted Spearman correlation that is used when the assumptions of the Pearson correlation are markedly violated (non-gaussianity). Spearman rho does not test the linear, but the ***monotonic*** relationship between bacterial abundances and cytokine levels.

As a consequence of the non-linearity of the relationship between bacterial abundances and cytokines, fitting a regression line over the scatter plots is not fully appropriate. A more appropriate approach is showing curves fitted through a data-driven approach, such as spline function estimation or local polynomial fitted curve (see e.g. *Cleveland WS, Grosse E, and Shyu WM, 1992, Local regression models. Chapter 8 of Statistical Models in S, eds Chambers JM and Hastie TJ, Wadsworth & Brooks/Cole*).

We have modified the scatter plots accordingly by adding the non-parametric fitted curves and we added this in the Statistical Analyses Section: *For the evaluation of correlations between stool bacteria and blood inflammation biomarkers, the algorithm for smoothing scatterplots by robust locally weighted regression (Cleveland, Grosse, Shyu, 1992) has been applied to draw the fitted curves*.

- The Abstract must be written more clearly, especially the paragraph dealing with cytokine levels. Is it really necessary to show all values in the abstract? The long list of percent values, p, values etc is difficult to read, in particular when there is one "Amy+ vs HC" in between that shall probably mean "only Amy+ vs HC". Not all abbreviations are explained (e.g. FC). Also the final paragraph should be modified. The authors did not study gut microbiota composition and whether it is pro- or anti-inflammatory. Instead they found that one putative pro-inflammatory taxon was increased in Amy+ vas May- and HC and one putative anti-inflammatory taxon was decreased. This does not tell us anything about the global functional output or immunological interactions of the whole microbiota. Correlations between the taxa and immune markers are very relevant if they remain significant after multiple comparison correction and in adjusted models (see above).

On a general level I would suggest to be cautious with uniformly designating certain bacterial taxa as pro- or anti-inflammatory since the effects of bacteria are dynamic and strongly dependent on the milieu. So I would suggest to use uniformly an expression like "putative" or "supposedly" when mentioning these activities as long as they have not been directly measured.

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We thank the reviewer for this suggestion. We have now revised all the manuscript and used the term taxon or taxa when appropriate.

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**Reviewer #3**

- The authors have adequately addressed my comments. One note: Figure 1 has both conditions labelled Ab-, whereas the black bars should be Ab+.

We are happy we have been able to address all the reviewer’s issues. Figure 1 has now been modified.

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*For Submission to Neurobiology of Aging*

**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 beta-amyloid deposition to cognitive impairment is believed to be a cornerstone of the pathogenesis of Alzheimer's disease (AD). However, what drives amyloid build-up in sporadic non-genetic 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 (i) GMB taxa with pro- and anti-inflammatory activity, and (ii) 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, IL-1β, IL-6, IL-18, IL-8, NLRP3, 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 controls (n=10, no brain amyloidosis and no cognitive impairment, HC).

Amy+ patients showed higher levels of pro-inflammatory cytokines (IL-6, CXCL2, NLRP3 and IL-1β) compared to both controls and to Amy- patients. A reduction of the anti-inflammatory cytokine IL-10 was observed in Amy+ versus Amy-. Amy+ showed lower abundance of *Eubacterium rectale* and higher abundance of *Escherichia/Shigella* as compared to both HC (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 *Eubacterium 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, *Eubacterium 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.

**Key words:** cognitive impairment, brain amyloidosis, inflammation, gut microbiota, neurodegeneration

## Introduction

Neurodegenerative disorders, including Alzheimer's disease (AD), are characterized by the accumulation in the brain of neurotoxic proteins. In AD, these are β-amyloid (Aβ) and hyper-phosphorylated 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 key in AD pathophysiology (Jack et al, 2013). In non-genetic 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 (Heppner et al., 2015; Clark et al., 2015; Heneka 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 (CSF) and blood (Vom Berg et al., 2012; Kauwe et al., 2014; Nascimento et al., 2014; Monson et al., 2014).

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 (CNS) 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 (Leung et al., 2013; Ray et al., 2007; Soares et al., 2012a; Doecke et al., 2012; Chen et al., 2015; 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 behaviour in rodents and humans (Li et al., 2009; Diaz et al., 2011; Bercik et al., 2011).

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 (Toumi et al., 2014; Luo et al., 2014). In Parkinson's disease, 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; Lebouvier et al., 2010; Goedert et al., 2013). 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 pathological processes associated with amyloidosis and AD (Asti & 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 & Gioglio, 2014; Schwartz  et al., 2013).

The aim of this study was to test, in elder patients with cognitive impairment, the association between brain amyloidosis and: (i) 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*) (Friedland et al., 2015; De la Fluente et al., 2014; Bruzzese et al., 2014; Cantarel et al., 2015); (ii) 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) (Leung et al., 2013; Ray et al., 2007; Doecke et al., 2012; Chen et al., 2015; Tan et al., 2013;Soares et al., 2012a).

## Methods

*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 INDIA-FBP study – Incremental Diagnostic value of Florbetapir Amyloid Imaging) (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 PET with $^{18}$F-Florbetapir. Two hundreds and forty-one patients and twenty-six 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 MRI and CSF analyses for Aβ and total and phosphorylated tau levels 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 n. 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 current study. Patients were defined as cognitively impaired in the case they matched these criteria: i) presence of cognitive complaints reported by patients or proxy or by the doctor; ii) presence of no intracranial metabolic or psychiatric causes of cognitive impairments; iii) presence of abnormal scores in two or more cognitive tests, and iv) 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

behaviour assessment, and neuropsychological assessment including the Mini Mental State Exam (MMSE) (Folstein et al., 1975). Body-mass index (BMI) was defined as height/weight$^2$ and measured in cm/kg$^2$. The neuropsychological battery consisted of tests tapping verbal and non-verbal 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 – MTA (Scheltens et al., 1993) and the Age-Related White Matter Changes – ARWMC – 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*.

*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-CT scanners, respectively. PET was a 10-minute (two 5-minutes 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 CT. PET images were reconstructed onto a 128x128 matrix with slice thickness of 3 to 3.3 mm, using a 2-3 mm Gaussian post reconstruction filter. Subjects were categorised into Amy+ and Amy- following a validated procedure (http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/PeripheralandCentralNervousSystemDrugsAdvisoryCommittee/UCM240266.pdf"). PET exemplars from a HC, an Amy+, and an Amy- participant can be found in Supplementary Material (*see Supplementary Figure 1*).

Amyloid positivity was visually rated independently by two 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.

*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 three times in three 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 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 0.5mg/mL ethidium bromide. DNA samples were then stored at -20°C until subsequent analyses.

*Bacterial DNA quantification in stools*

The abundance analyses of the selected bacterial taxa (*Escherichia/Shigella, Pseudomonas aeruginosa*, *Eubacterium rectale, Eubacterium hallii, Faecalibacterium prausnitzii* and *Bacteroides 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 manufacturer's instructions. Fifty nanograms of total DNA were 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 above 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; *B. fragilis:* BPID00146AR).

The abundance of each taxon was then calculated according to the comparative Ct method (-ΔΔCt method) (Schmittgen et al., 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.

*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 analysed by a 384 wells qRT-PCR instrument (Bio-Rad Instrument), using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories) and Applied BioSystem 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 three housekeeping genes (HK), 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 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 et al., 2008), where the control subjects (HC) have been used as reference group. We thus obtained, as output, a Relative Expression Ratio (R) value for each cytokine in each sample.

*Statistical analysis*

Parametric (ANOVA, t-test and Chi-square) and non-parametric (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, i.e. 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 (AIC and BIC index 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, IQR) and QQ-plot inspection and tested by Shapiro-Wilk and Kolmogorov-Smirnov tests. According to the distribution of dependent variables (bacteria taxa abundances or cytokines mRNA levels) we then applied: (i) Generalized Linear Models (GLM), with log link function for the Gamma distributed data, for the evaluation of all bacteria taxa across groups; and (ii) Analysis of Covariance (ANCOVA) models for cytokine blood levels. GLM and ANCOVA 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.

## Results

*Clinical sample description*

The three 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 any significant difference on medial temporal atrophy (Scheltens' scale: mean ± SD 1.8±1.1 and 2.0±0.8; p=0.670) nor white matter changes (ARWMC scale: mean ± SD 2.5±3.1 and 1.8±2.2; p=0.520).

*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, assumption of cholinergic drug) variables. We found a significant association only between *Pseudomonas aeruginosa* and BMI (rho=0.41, p=0.037). Thus, BMI was included, together with the MMSE, as a covariate in the GLM with *Pseudomonas aeruginosa* as dependent variable. No correlations with age (p values of Spearman correlation >0.097) as well as 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 zero and a continuous, right-skewed distribution elsewhere indicating a Gamma distribution (p-values of Kolmogorov-Smirnov test for Gamma distributions larger than 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 *Pseudomonas aeruginosa*'s analysis which was adjusted also for BMI.

In Amy+ we found a specific pattern of alterations in bacteria taxa abundance when compared to HC and to Amy+. In particular, Amy+ showed lower abundance of *Eubacterium rectale* and higher abundance of *Escherichia/Shigella* as compared to 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 *Bacteroides 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 (**Figure 1**).

*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 larger than 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.

As shown in **Figure 2**, Amy+ showed a specific pattern of higher levels of four pro-inflammatory cytokines (NLRP3, CXCL2, IL-6 and IL-1β), as their levels were different compared to 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; +22%, p=0.040).

Amy+ and Amy- showed a similar pattern of increased expression of TNF-α when compared to 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 to 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).

*Correlation between stool bacteria and blood inflammation biomarkers*

We performed Spearman correlation analyses between cytokines and bacteria strains focussing 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, IL-10 with the stool abundance of *Escherichia/Shigella* and *Eubacterium rectale*. We found a positive correlation between 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) (**Figure**

**3**). A negative correlation was observed between blood levels of the pro-inflammatory cytokines IL-1β, NLRP3 and CXCL2 with stool abundance of anti-inflammatory bacteria *Eubacterium 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 *Eubacterium rectale* (rho=0.30, p=0.030) (**Figure 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 *Eubacterium 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).

## 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 *Eubacterium 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, as well as reduced levels of the anti-inflammatory cytokine IL-10. Interestingly, the abundance of *Escherichia/Shigella* correlated positively with the levels of IL-1β, CXCL2, NLRP3, whereas *Eubacterium 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 neuropathological studies have suggested a possible involvement of GMB composition in AD pathogenesis. Recent data show that bacterial endotoxins may exert a key role in the inflammatory and pathological processes associated with amyloidosis and AD (Asti, 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, Gioglio, 2014; Schwartz et al., 2013). Recently, Kamer and collaborators (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 to Amy- patients. *Escherichia/Shigella* has been associated with a pro-inflammatory status (Morgan et al., 2013; Soares et al., 2012b) and, in a recent study, Small and collaborators (Small et al., 2013) found that persistent infection with adherent and invasive *Escherichia* led to chronic and persistent

peripheral inflammation. Also De La Fuente and collaborators reported an ability of the genus *Escherichia* to induce the production of pro-inflammatory cytokines through NLRP3-dependent mechanism (de la Fluente et al., 2014). Interestingly, in line with the studies mentioned above, 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 *Eubacterium rectale* abundance in Amy+ as compared to Amy- subjects. *Eubacterium 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 *Eubacterium rectale* abundance is associated with lesser degree of inflammation. An increase in *Eubacterium 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 multiple sclerosis (MS) and Parkinson's disease (PD) that share with AD neuroinflammation and protein misfolding, respectively. In MS, studies in animal models and in germ-free mice (Forsythe et al., 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 (Scheperjans et al., 2015; Keshavarzian et al., 2015; Hasegawa 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 (Keshavarzian et al., 2015; Hasegawa 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 Keshavarzian's study, 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 as compared to PD patients. Moreover, an increase of *Faecalibacterium* and a reduction of *Proteobacteria* were found in the mucosa of controls compared to PD patients. In the other study, Hasegawa and colleagues measured serum markers and quantified 19 fecal bacterial taxa by qPCR in a group of PD patients compared to healthy controls. The authors found that the abundance of *Lactobacill*i was higher whereas those of *Clostridium coccoides* and *Bacteroides 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 ALS-dementia complex of Guam (Cox et al., 2003) due to N-β-methylamino-L-alanine (BMAA) 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 (NGS), but we have investigated 6 specific taxa. However, if the added value of NGS is to identify, by using a hypothesis free approach, all the possible taxa differentially modulated in a pathological 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 (Holmes et al., 2012; Cryan 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, *Eubacterium 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.

## Legends to Tables and Figures

**Table 1: Demographic and clinical features of study participants.**

Numbers denote mean ± standard deviation. p indicates the significance of the group differences on Students' t or chi-square test.

**Figure 1. Abundance of bacterial taxa in the stools of study participants.**

Bars denote Fold changes (FC) 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 zero), according to the ΔΔCt method (for details see methods). * Statistical significance at $p<0.05$, ** at $p<0.01$ and *** at $p<0.001$ when comparing Amy+ and Amy- vs HC; # Statistical significance at $p<0.001$ when comparing Amy+ vs Amy-.

**Figure 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), amyloid positive (Amy+) and amyloid negative (Amy-) patients. Bars denote mean ± standard error. * Statistical significance at $p<0.05$ and ** at $p<0.001$ when comparing Amy+ or Amy- vs HC; # Statistical significance at $p<0.05$ when comparing Amy+ vs Amy-.

**Figure 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, 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%.

**Figure 4. Spearman correlation of cytokines blood levels with *Eubacterium rectale* abundance in the stools in study participants.**

Cytokines (in the y axis) and *Eubacterium rectale* abundance (in the x axis) were selected based on significance of the difference between groups. Full dots denote control subjects, 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%.

**Supplementary Figure 1: PET scans with $^{18}$F-Florbetapir in exemplary study participants.**

Tracer uptake is represented with a color map superimposed on the individual CT scan of exemplary cases. Unspecific tracer uptake confined to the white matter is present in control subjects and Amy- patients (left and middle panels). Amy+ patients feature, in addition to unspecific uptake, specific tracer uptake in the cortex (right panel).

**Supplementary Table 1: Psychological assessment evaluation in study participants.**

Assessment included evaluation of:

- *global cognition* (Mini-Mental State Examination MMSE, and Alzheimer's Disease Assessment Scale – Cognitive ADAS-COG);
- *long-term memory* (Story Recall Test, Rey Auditory-Verbal Learning Test – immediate and delayed recall (Rey AVLT), and Recall of Rey-Osterrieth Complex Figure);
- *attention* (Trail Making Test – Part A TMT-A);

- *language* (Token Test, Action and Object naming -subtest from the Battery for Analysis of Aphasic Deficits, BADA-, and Letter and Category Fluency);
- *constructional and visuo-spatial abilities* (Copy of Rey-Osterrieth Complex Figure);
- *upper limb apraxia* (Movement imitation Test);
- *executive functions* (Trail Making Test – Part B (TMT-B), and Wisconsin Card Sorting Test (WCST));
- *non-verbal reasoning* (Raven's Coloured Progressive Matrices (Raven's CPM)).

Numbers denote the mean values of test results ± standard deviation.

**Supplementary Table 2: List of current medications in study participants.**

Table show the current medications in Amy+ and Amy- patients with numbers denoting percentage (%) of subjects.

## Acknowledgements

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Figure 1
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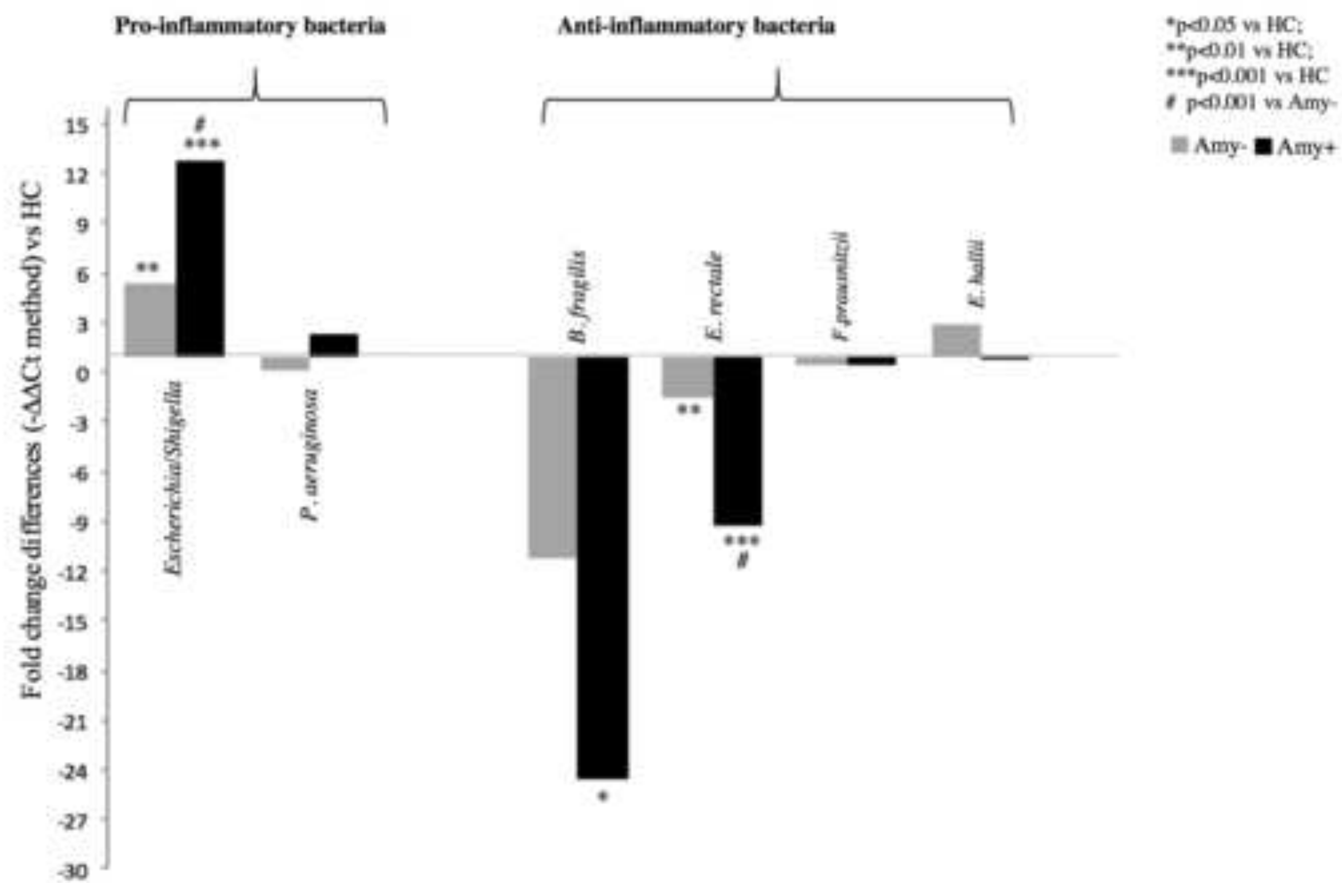


**Figure 1** Abundance of bacterial taxa in the stools of the study participants.

Figure 2
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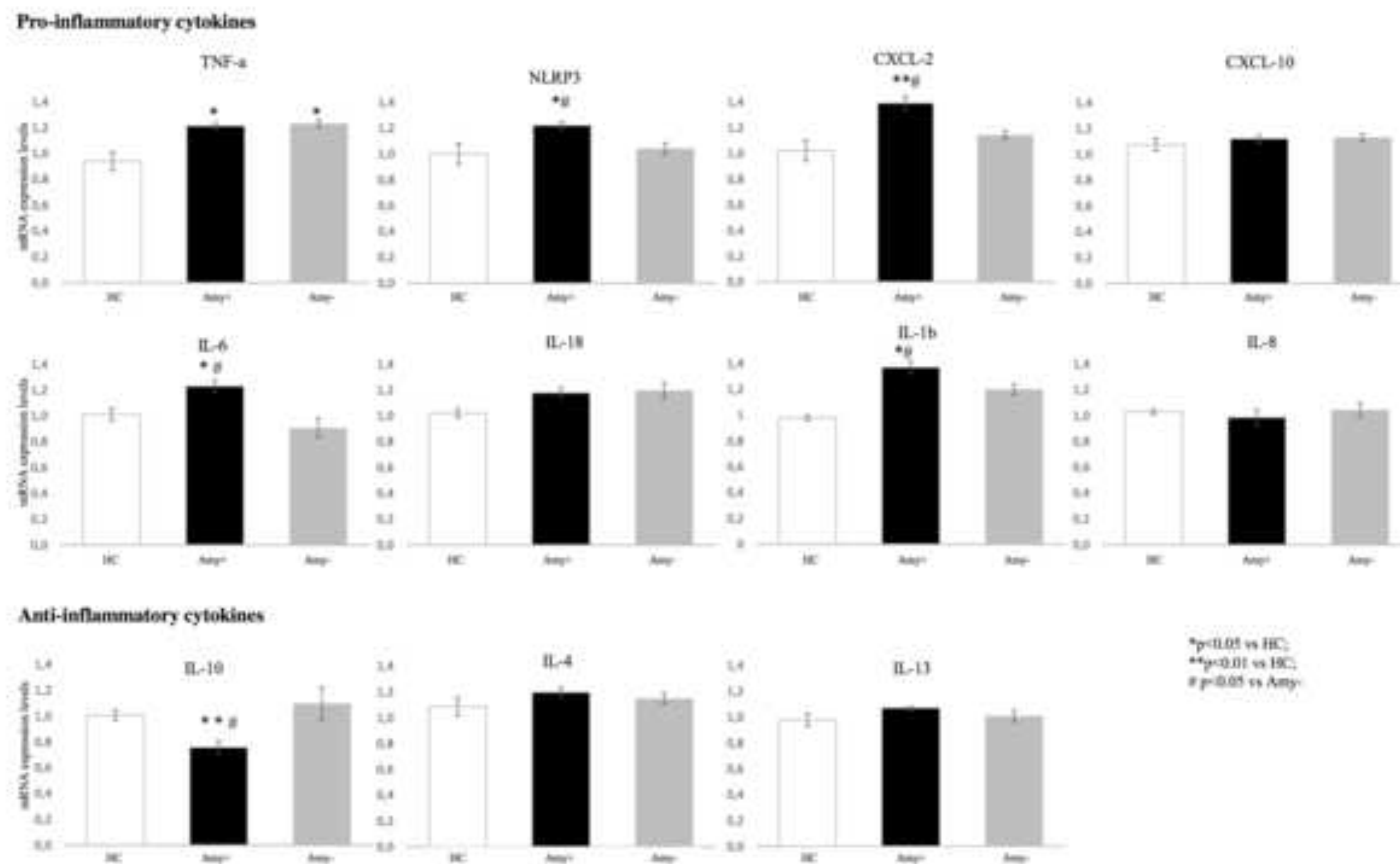


**Figure 2** Expression levels of inflammation-related cytokines in the blood of study participants

Figure 3

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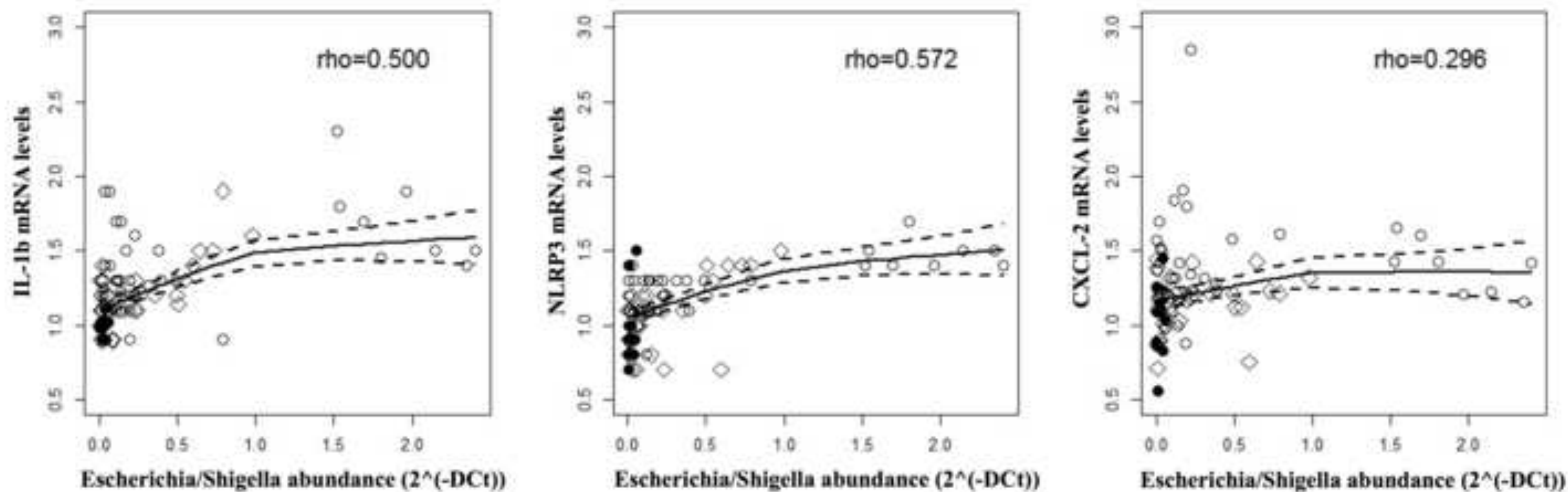


**Figure 3**

Spearman correlation of cytokines blood levels with *Escherichia/Shigella* stool abundance in study participants.

Figure 4
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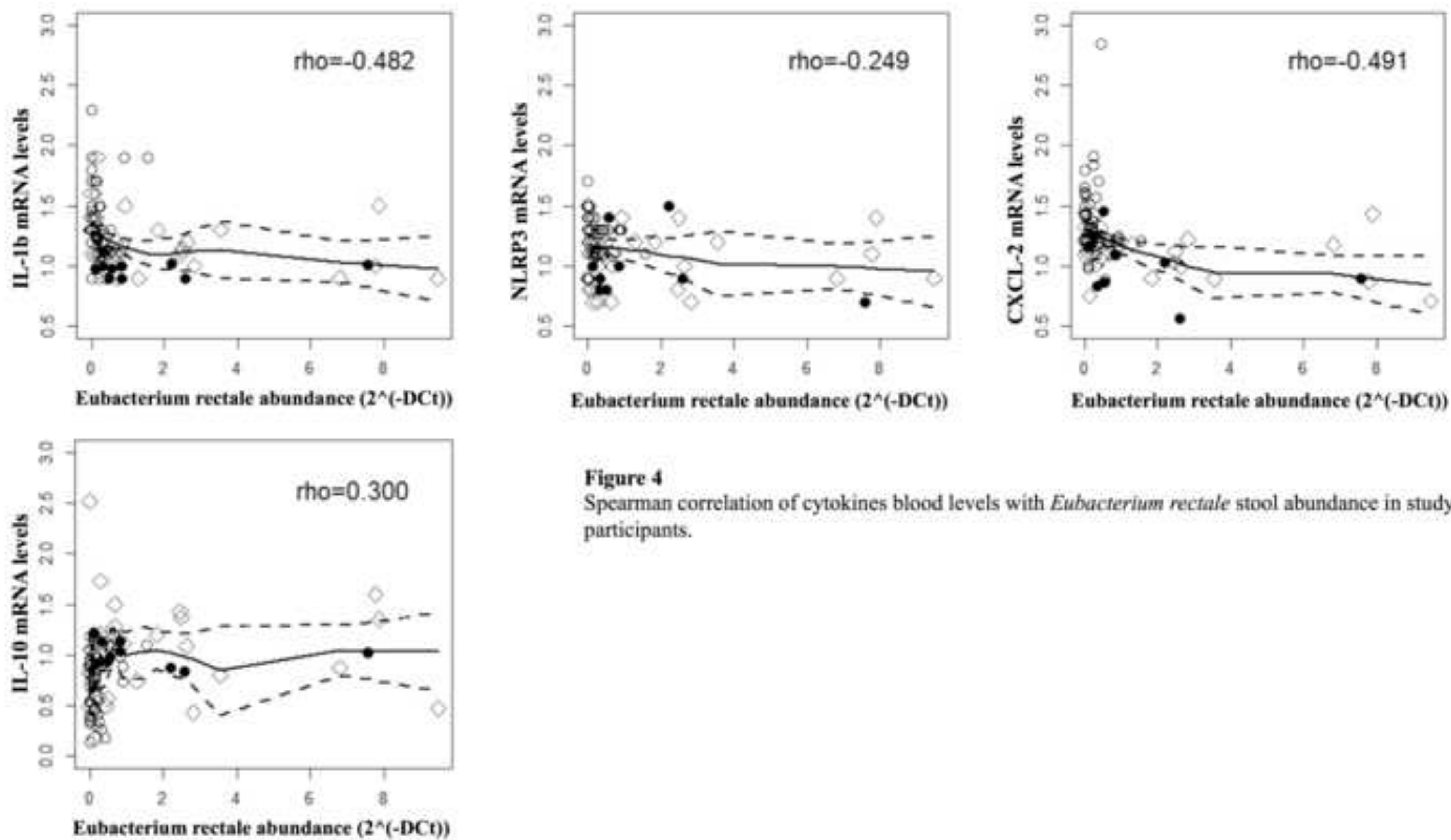


**Figure 4**
Spearman correlation of cytokines blood levels with *Eubacterium rectale* stool abundance in study participants.

Table 1

| | **Amy+ (n=40)** | **Amy- (n=33)** | **HC (n=10)** | **Significance (p)** |
|---|---|---|---|---|
| **Age (years)** | 71 ± 7 | 70 ± 7 | 68 ± 8 | n.s. |
| **Females** | 20/40 | 18/34 | 6/10 | n.s. |
| **Body Mass Index** | 24.5 ± 3.5 | 25.6 ± 3.7 | 24.3 ± 2.9 | n.s. |
| **Mini Mental State Exam** | 21.3 ± 6.1 | 25.5 ± 3.9 | 28.3 ± 1.1 | $p<0.01$ Amy+ vs Amy-; $p<0.05$ Amy+ vs HC |

**Table 1: Demographic and clinical features of study participants.**

**Supplementary Table 1**

| | | Patients AMY negative (n=33) | Patients AMY positive (n=40) | HC AMY negative (n=10) |
|---|---|---|---|---|
| GLOBAL COGNITION | MMSE | 25.52 ± 3.87 | 21.30 ± 6.09 | 28.30 ± 1.06 |
| | ADAS-COG | 14.47 ± 9.75 | 19.93 ± 10.68 | 7.63 ± 2.25 |
| MEMORY ASSESSMENT | Story Recall Test | 8.14 ± 4.78 | 4.82 ± 4.34 | 10.40 ± 2.80 |
| | Immediate Rey AVLT | 32.03 ± 9.19 | 26.27 ± 11.10 | 39.80 ± 8.12 |
| | Delayed Rey AVLT | 5.24 ± 2.94 | 2.33 ± 2.78 | 7.90 ± 2.73 |
| | Recall of Rey-Osterrieth Complex Figure | 8.40 ± 5.03 | 4.82 ± 5.40 | 13.30 ± 3.26 |
| LANGUAGE | TOKEN TEST | 29.85 ± 4.40 | 27.65 ± 5.81 | 33.20 ± 1.42 |
| | BADA (object naming) | 25.87 ± 4.27 | 25.67 ± 2.99 | 28.20 ± 1.23 |
| | BADA (action naming) | 23.52 ± 3.49 | 21.57 ± 4.40 | 21.50 ± 1.72 |
| | Category Fluency | 29.74 ± 9.33 | 27.53 ± 10.49 | 36.80 ± 14.43 |
| | Letter Fluency | 25.61 ± 8.83 | 30.07 ± 12.98 | 39.60 ± 8.46 |
| Constructional and visuo-spatial abilities | Copy of Rey-Osterrieth Complex Figure | 27.62 ± 6.38 | 25.30 ± 10.00 | 30.50 ± 2.71 |
| Upper Limb apraxia | Movement imitation Test (DX) | 64.80 ± 5.10 | 62.33 ± 7.53 | 67.50 ± 3.14 |
| | Movement imitation Test (SX) | 67.31 ± 4.48 | 63.31 ± 8.84 | 69.50 ± 2.88 |
| Executive Function | WCST (GLOBAL SCORE) | 79.33 ± 35.28 | 89.10 ± 33.02 | 105.00 ± 11.60 |
| | TMT-A | 65.06 ± 34.75 | 95.55 ± 90.99 | 43.70 ± 22.38 |
| | TMT-B | 189.88 ± 95.93 | 217.53 ± 121.32 | 133.60 ± 69.12 |
| | TMT(B-A) | 137.59 ± 81.04 | 151.06 ± 107.15 | 89.90 ± 63.18 |
| Non-verbal reasoning | Raven's CPM | 21.85 ± 6.12 | 17.92 ± 9.39 | 27.40 ± 5.42 |

**Supplementary Table 1**

**Psychological assessment evaluations in study participants.**

**Supplementary Table 2**

| | Amy- (n=33) | | | Amy+ (n=40) | | |
|---|---|---|---|---|---|---|
| | yes | no | n.a. | yes | no | n.a. |
| **Anticholinesterases and anticholinergic drugs** | 3 (9.1%) | 29 (87.9%) | 1 (3%) | 24 (60%) | 13 (32.5%) | 3 (7.5%) |
| **NMDA Receptor Antagonists** | 0 (0%) | 32 (97%) | 1 (3%) | 8 (20%) | 29 (72.5%) | 3 (7.5%) |
| **Antihistamines** | 0 (0%) | 32 (97%) | 1 (3%) | 2 (5%) | 35 (87.5%) | 3 (7.5%) |
| **Dyslipidemia drugs** | 3 (9.1%) | 29 (87.9%) | 1 (3%) | 7 (17.5%) | 30 (75%) | 3 (7.5%) |
| **Hypertension medications** | 5 (15.2%) | 27 (81.8%) | 1 (3%) | 11 (27.5%) | 26 (65%) | 3 (7.5%) |
| **Antipsychotics** | 2 (6.1%) | 30 (90.9%) | 1 (3%) | 3 (7.5%) | 34 (85%) | 3 (7.5%) |
| **Benzodiazepines** | 5 (15.2%) | 21 (63.6%) | 7 (21.2%) | 7 (17.5%) | 27 (67.5%) | 6 (15%) |
| **Antidepressants** | 9 (27.3%) | 17 (51.5%) | 7 (21.2%) | 16 (40%) | 18 (45%) | 6 (15%) |
| **Hypoglycemic drugs** | 1 (3%) | 31 (94%) | 1 (3%) | 1 (2.5%) | 36 (90%) | 3 (7.5%) |
| **Gastroprotective drugs** | 2 (6.1%) | 30 (90.9%) | 1 (3%) | 5 (12.5%) | 32 (80%) | 3 (7.5%) |
| **Anticoagulants** | 1 (3%) | 31 (94%) | 1 (3%) | 4 (10) | 33 (82.5%) | 3 (7.5%) |

**Supplementary Table 2**
**List of concomitant medications in Amy+ and Amy- patients. Numbers denote % of patients in each group (yes/no/not available)**

Supplementary Figure 1
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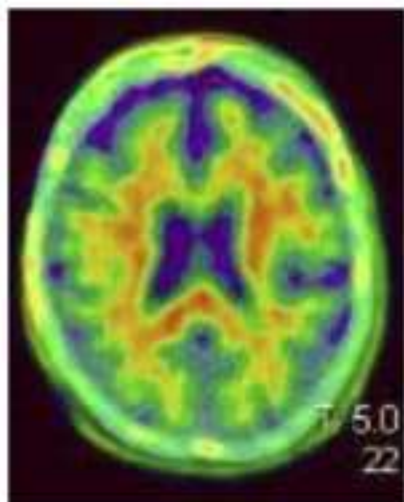


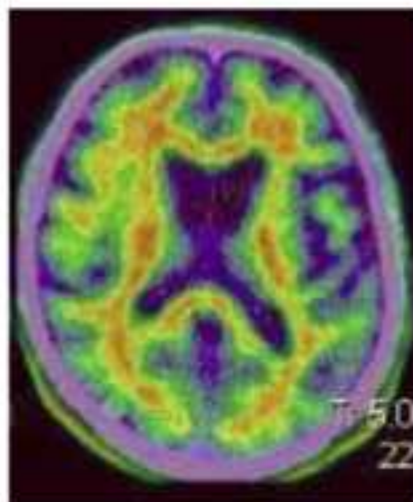


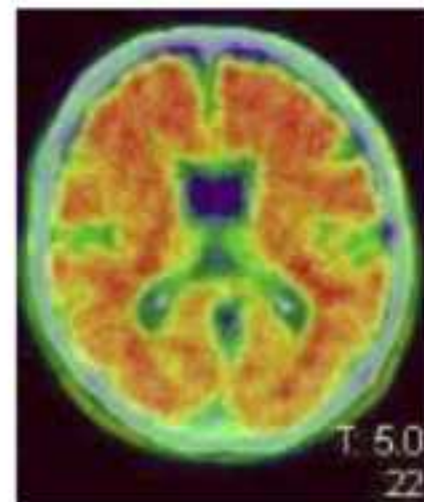


**HC**
Amyloid negative

**MCI**
Amyloid negative

**MCI**
Amyloid positive

**Supplementary Figure 1**
**PET scan images with 18F-Florbetapir in a control subject and in patients with cognitive impairment amyloid-negative and –positive.**