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Detection of fecal amyloid in subjects with early stage Alzheimer's disease

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Abstract

Introduction: Alzheimer's disease (AD) is the most frequent form of dementia in the Western world and its prevalence is estimated at 4.4% in the over 65 years and more than 25% in the over 90 years. It is characterized by the deposition of beta amyloid and tau peptides in the brain. To help with clinical diagnosis (neuropsychological evaluation, functional and psychiatric endpoints), CSF biomarkers, by detecting A β and Tau peptides, are among the only one available. Peripheral biomarkers, such as blood, have all failed to show consistency. However, it has been shown that A β peptide is able to leave the brain toward the blood system and is then transported to peripheral organ. The mechanism by which the peptide is cleared from peripheral organs is not completely known yet, but one hypothesis is that it is partly eliminated by bile excretion in the stools.

Objectives and methods: The study consists of detection and quantification of A β 42 peptide in the stools of people suffering from AD and control non-demented individuals. The objective is to evaluate a new tool that should allow diagnosis or screening of AD in waste products. We analyzed amyloid levels in stools of 9 subjects with AD and 9 non-demented age-matched control subjects. We also exposed stool amyloid levels detected in 4 amyloid-depositing transgenic mice expressing AD, APPPS1 mice. Detection and quantification of A β 42 peptide were performed using a sandwich ELISA technique.

Results: Fecal A β 42 levels tend to be higher in Alzheimer's subjects compared to control subjects (mean value 14.54 ng/l and 10.10 ng/l respectively, AD to control ratio 1.44, p = 0.015), in dementia subjects compared to MCI subjects (mean value 16.8 ng/l and 12.74 ng/l respectively, p = 0.254), in dementia subjects compared to control subjects (p = 0.011) and in MCI subjects compared to control subjects (p = 0.156). Every correlation analysis made did not show any significant results.

Discussion: In this study, we showed that detection of A β 42 peptide in human stools using ELISA technique was possible and that A β 42 rates were higher in AD subjects than control individuals without significant brain amyloidosis (AD to control ratio 1.44). However, larger scales studies are needed to confirm those results and to evaluate the correlation between fecal A β levels and other clinical or biological biomarkers of AD. Nevertheless, the use of stool as a diagnostic tool for AD is promising. Yet, due to difficulties concerning the stool harvest process in relation to cognitive impairment, this test could only be offered to early stage Alzheimer's patients (MCI stage) or be performed by caregivers in institutionalized subjects in a medico-social institution.

Key words

Alzheimer's disease, fecal, stool, beta-amyloïd, diagnosis

1. Introduction

1.1 Alzheimer's disease [1,2]

Alzheimer's disease (AD) is the most frequent form of dementia in the Western world, accounting for more than 60% of cases [3]. A European meta-analysis evaluates its prevalence to 4.4% in the over 65 years and more than 25% in the over 90 years [4]. It is characterized by a progressive degeneration of neurons, the one affected the earliest being those located in the internal temporal region, mainly the hippocampus. This neuronal loss leads to an irreversible loss of cognitive functions, especially memory and attention. Subsequently, degeneration extends to other cerebral areas, mainly the parieto-temporal associative cortex, leading to more variable cognitive disorders.

The cause of the neurodegeneration in AD is still poorly understood but we know that it is strongly associated with two brain lesions that are characteristic of the disease: senile plaques (or amyloid plaques) and neurofibrillary tangles (NFT). Senile plaques are extracellular deposits made of beta amyloid (A β) peptide surrounded by degenerative neuronal extensions and inflammatory cells such as microglia and astrocytes. NFT results from the accumulation inside the neurons of microtubule-associated tau protein, mainly its hyperphosphorylated form called phospho-tau (p-tau). These two lesions are probably the cause of the neurodegeneration that characterizes the disease and most authors seem to agree that amyloid plaques play an early and critical role in the pathogenic cascade leading to cell death and dementia.

Classically, the evolution of the disease can be divided into three phases. A preclinical asymptomatic phase where neuropathological processes have nevertheless already begun. This phase probably lasts for decades and goes completely unnoticed [5]. A predemential phase, or mild cognitive impairment (MCI), in which the patient presents a cognitive decline but without being sufficient to impact his autonomy. Finally, a demential phase in which the cognitive impairment is severe enough to affect patient's autonomy and daily life activities. Memory loss is usually among the first cognitive symptom of the disease, a learning and recall of recently introduced element disability. With the evolution of the disease, other cognitive impairments may appear such as aphasia, visual agnosia, apraxia, executive dysfunction, and neuropsychiatric disorders.

The diagnosis of AD is currently essentially clinical, based on medical records, neurological examination, and neuropsychological evaluation. Diagnostic criteria for probable AD are derived from National Institute on Aging-Alzheimer's Association (NIA-AA) guidelines [6]. Some biomarkers of the disease are available and can improve diagnostic performance (see chapter 1.2), but to date, postmortem neuropathology remains the gold standard, the only exam that can establish for sure the diagnosis by highlighting the presence of senile plaques, NFT and neurodegeneration in the brain. Unfortunately, this exam can only be carried out after the death of the affected person.

1.2 Biomarkers of AD

Biomarkers provides a biological measure of the underlying physiopathology of AD. A limited number of them are available that can increase sensitivity and specificity in the diagnosis. They include imaging and biochemical biomarkers. Structural MRI (Magnetic Resonance Imaging) can show brain atrophy, mainly in the internal temporal region and is therefore a biomarker of AD-related neurodegeneration. [¹⁸F] FDG PET (fluorodeoxyglucose position emission tomography) scan shows decreased glucose metabolism in parieto-temporal cortex and is therefore a biomarker of synaptic dysfunction [7]. Recently, a new imaging modality has emerged, named amyloid PET. This imaging uses a ligand capable of binding directly to the A β peptide and can therefore visualize and quantify it; the most widely used radiotracer is [¹¹C] PiB (Pittsburgh compound B) [8].

On the other hand, some biochemical biomarkers are available, and the best one so far remain the detection and quantification of A β , total-tau (t-tau) and phospho-tau (p-tau) peptides in the cerebrospinal fluid (CSF). CSF is in direct contact with the brain and therefore gives valuable information about cerebral biochemical changes in [9]. In AD, CSF A β is decreased while CSF t-tau and p-tau are increased [10]. It has been shown that changes in concentration of A β peptide in the CSF can already be detected about 25 years before the first symptoms, in the preclinical phase of the disease [11]. Unfortunately, CSF collection requires a lumbar puncture (LP) and cannot be performed routinely. Some peripheral biomarkers have been evaluated, such as detection of A β or tau peptide in the blood, but results were inconsistent [12,13]. To date, there is no peripheral biochemical biomarkers of the disease, either in the blood, urine, saliva, or stool. A temporal model of AD including biomarkers is shown in figure 1.



FIGURE 1 Temporal model of AD integrating biomarkers

The black horizontal line represents the threshold for the first detection of biomarkers of AD. The gray area is the zone in which pathological changes occurs but remains below the detection threshold. Aβ deposition, detected in the CSF or by amyloid PET is the earliest biomarker that rises above detection threshold. It is then followed by CSF tau, MRI and FDG PET. It is only later cognitive impairment that evident, evolving becomes from MCI to dementia.

1.3 Senile plaques and amyloid peptide metabolism [15-18]

Senile plaque is one of the pathological lesions that is characteristic of AD but not pathognomonic. This lesion, located in the extracellular matrix of the brain, can be divided into two parts: a center consisting of aggregates of the β amyloid peptide and a periphery containing degenerating neurons, astrocytes, and microglia. Diffuse plaques also exist and miss a clear defined amyloid core.

The Aß peptide is naturally produced by the human body, especially in the brain by neurons, astrocytes, and microglia, but also by peripheral tissues (figure 3) [19]. Its production from the precursor Amyloid Precursor Protein (APP) (figure 2) generates peptides of variable size named according to their number of amino acids, between 38 and 43. The most abundant are Aβ40 (80-90%) and Aβ42 (5-10%). Under physiological conditions, a steady state exists between production and clearance of the peptide which prevents its accumulation. Aß is cleared from the brain by several mechanisms: phagocytosis by microglia, macrophages, astrocytes, and neurons; degradation by various enzymes such as insulin-degrading enzyme; and efflux to the peripheral circulation via transporters across the blood-brain barrier (BBB), the blood-CSF barrier and interstitial fluid flow (figure 3) [20]. During physiological aging or in AD, this steady state is broken. The excessive production of the peptide or its insufficient clearance causes its accumulation and aggregation in the cerebral parenchyma. AB42 is the most hydrophobic and fibrillogenic form and has a particular tendency to aggregate; this is the most abundant form in senile plagues. In sporadic forms of AD, the etiology of this overproduction of amyloid peptide is poorly understood and appears to be multifactorial, with environmental [21] and genetic risk factors such as £4 allele of Apolipoprotein E [22]. Genetic forms of AD on the other hand, are linked to mutations of the APP, PSEN1 (y-secretase subunit) and PSEN2 (y-secretase subunit) genes, genes that are involved in the production of Aß peptide and causing overproduction or hyperaggregation of this one (figure 2) [23].



FIGURE 2 Amyloidogenic and non-amyloidogenic pathways of APP (amyloid precursor protein) metabolism [24]

Metabolism of APP peptide is split in two pathways. 1) The non-amyloidogenic pathway, representing 90% of the APP peptide metabolism, is carried out by an α-secretase and then y-secretase. 2) The а amyloidogenic pathway, representing 10% of the APP peptide metabolism, is carried out by a β-secretase followed by a y-secretase and results in the production of the Aß peptide.

Senile plaques are generally referred to as the main cause of neuronal degeneration in AD. A β has shown to promote neuronal toxicity by several mechanisms such as disruption of intra-neuronal homeostasis [25], excitotoxicity by activation of NMDA receptors [26], immunity induction [27] but also by participating in the production of NFT [28].

A large body of evidence suggests that A β , tau and many other proteins involved in neurodegenerative disorders share common characteristics with prions [29,30]. Prion is non-living infectious agent responsible for transmissible spongiform а encephalopathies, such as Creutzfeldt-Jakob disease. The physiological prion (PrP^c) is a protein normally present in our cells. The pathological prion (PrP^{sc}) is a poorly folded variant of the physiological prion. The pathological prion can contaminate other prions, namely to induce their bad folding, causing its accumulation in the tissue and the formation of plaques. According to some authors, amyloid peptide would behave in a similar way and would be capable of contaminating other AB peptides and change their conformation, explaining the dissemination of the peptide in the CNS. Some also assume an initial production of the peptide in the digestive tract and its transmission to the CNS using the same route as the prion [31]. These evidences therefore suggest that Aß peptide has a "prion-like" behavior and reclassify AD as a "prion-like disease" [32]•

It has been shown that cerebral AB can leave the brain toward the blood system through several mechanisms [20,33]. In the blood, some clearance mechanisms exist, such as phagocytosis by monocytes and neutrophils and enzymatic degradation, similar to the mechanism present in the brain [34]. But AB is also transported to peripheral organs by erythrocytes, albumin, and lipoproteins [35,36]. Injection of iodinelabeled Aß peptide through the venous system of transgenic mice showed the presence of the peptide in various organs, especially the liver, kidney, gastrointestinal tract, and skin_[37]. Yet, the mechanism by which the peptide is cleared from peripheral organs is not completely known. The main mechanisms would be phagocytosis by macrophages and hepatocytes followed by subsequent excretion via bile or urine [38]. Excretion via bile into stool is thought to be one of the major mechanism by which AB is eliminated from periphery, we can therefore assume that the peptide could be detected in human stools. This hypothesis is supported by a preclinical study carried out on transgenic mice expressing the human $A\beta$ protein under the control of an exclusively neuronal promoter (APPPS1 mice, see chapter 3.4). In this study, it was possible to detect the presence of Aß peptide in stool of these mice expressing a genetic form of AD (Bolmont et al., in preparation). It is therefore probable that the AB peptide is also detectable and quantifiable in the stools of humans with a sporadic form of AD. Figure 3 resume A β production and degradation in the brain and in periphery.



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FIGURE 3 Physiological metabolism of Aβ in the brain and periphery [18]

In the brain, $A\beta$ is generated by neurons, astrocytes, and microglia. In the periphery, it is generated by platelets, osteoblasts, skin fibroblasts and skeletal muscle cells. In the brain, $A\beta$ is cleared by several mechanisms: phagocytosis by microglia, macrophages, astrocytes, and neurons; enzymatic degradation; and efflux to the peripheral circulation via transporters in the BBB, blood-CSF barrier, and interstitial flow clearance. In the blood, $A\beta$ is cleared by phagocytosis by monocytes or neutrophils, degraded by $A\beta$ degrading enzymes and transported, by red blood cells, lipoproteins, or albumin, in the peripheral tissues. Here, $A\beta$ is degraded by macrophages or hepatocytes, or excreted with bile or with urine.

Peripheral A β could also enter the brain by some transporters, such as RAGE, and participate to AD pathology.

2. Hypothesis and objectives

Since there is currently no available peripheral biomarker of AD, the research for marker in the stool seems to be promising avenue. This study consisted of the detection and quantification of the A β 42 peptide in the stools of people suffering from AD and its comparison with controls subjects. The following hypothesis have been made:

- Amyloid peptide circulates outside the brain, in the blood and is transported to peripheral organs, including the liver, and is then eliminated in the stool by bile. It is therefore possible to detect the peptide in the stools of people suffering from AD.
- The amount of amyloid peptide present in the stool is indicative of the presence and amount of amyloid peptide present in the brain of subjects suffering from AD.
- \circ The amount of amyloid peptide present in the stool is inversely correlated with the amount of amyloid peptide present in the CSF. Indeed, due to parenchymal sequestration of Aβ in the brain, its concentration is found to be reduced in the CSF.
- The amount of amyloid peptide present in the stool is indicative of the presence and degree of evolution of AD. There is therefore an inverse correlation between fecal amyloid peptide level and cognitive performances evaluated by neuropsychological screening tests such as MoCA (Montreal cognitive assessment).

The objectives of this study were multiple. We in fact evaluated a new potential diagnosis tool that should allow the detection and quantification of the amyloid peptide Aβ42 in the stools of people with AD. From a scientific point of view, the possibility of detecting Aβ42 in the stools would improve our understanding of the physiopathology of this very complex disease. It would support the hypothesis that this peptide leaves the brain for the periphery (or inversely according to the "prion-like" hypothesis) and that it is eliminated from the peripheral tissues, mainly the liver, in the stool. From a clinical point of view, the benefits would be multiple. Currently, AB quantification has shown clinical benefit only in the CSF. Trials for the detection of AB in the blood and other biological fluid have been carried out but have not been conclusive. This tool would therefore allow a noninvasive and quantitative molecular diagnosis of AD, stool harvest being significantly less invasive than the lumbar puncture required for cerebrospinal fluid collection. The simplicity of such a harvesting process has been evaluated in a population of subjects with cognitive impairment, which can make the process much more complex. At the same time, this tool would allow the possibility of screening for AD in stools, just like the screening for colorectal cancer. We know indeed that the detection of amyloid beta biomarkers precedes by several decades the appearance of clinical symptomatology (figure 1). Of course, the ethical issues of a possible screening for AD in the population would have to be discussed if necessary.

3. Subjects and methods

3.1 Study characteristics

From November 2015 to January 2018, we conducted a prospective clinical study at the Leenaards Memory Center (CLM), department of clinical neurosciences, CHUV, Lausanne, Switzerland. This study involved the analysis and comparison of amyloid A β 42 levels in the stools of people suffering from AD those of non-demented persons. We also exposed results of a previous study conducted by Bolmont et al., which consisted of detection and quantification of amyloid levels in stools of transgenic models of the disease, APPPS1 mice. This study has been approved by the cantonal human research ethics commission of Vaud (CER-VD), Switzerland.

3.2 Subjects selection

We decided to compare and analyze amyloid levels in stools of twenty subjects: ten subjects suffering from AD and ten non-demented aged control subjects. Subjects with AD were recruited at the Leenaards Memory Center. We recruited subjects that would fulfilled these three criteria: a diagnosis of probable AD according to the NIA-AA criteria [6], a neuropsychological assessment carried out and compatible with AD and the place of residence of the subject being in the state of Vaud, Switzerland. Subjects with AD in the mild cognitive impairment stage were included provided that the diagnostic suspicion was sufficiently strong. We did not retain brain imaging nor the research for biochemical biomarkers in the CSF as inclusion criteria. The subjects all benefited from a neuropsychological screening test such as the MoCA or the MMSE (Mini Mental State Examination) in the year prior to inclusion. The exclusion criteria are summarized in table 1. We contacted those potentially compatible subjects during their medical appointment at the CLM and if they fulfilled inclusion and exclusion criteria, the participation in the study was proposed to them. For the shake of convenience, we proposed the stool collection to be carried out at their home, using a supplied equipment (the same equipment as used for colorectal cancer screening in Switzerland).

Thirty-four subjects with AD, who fulfilled inclusion and exclusion criteria, were proposed to participate. Seven of them refused and twenty-seven accepted. Among these, seventeen did not continue their participation in the study, mainly because of difficulties with the stool collection process. We noticed that a stool collection, performed at home by the person itself, was difficult in people with major cognitive, especially memory, impairment. Control samples were obtained in a healthy aging (>55 years) and non-demented population and had no evidence of cognitive impairment. Free and informed consent of each subject, Alzheimer's, and control, was collected prior to inclusion in the study.

Presence of a positive HIV-1 viremia in the 12 months preceding the study

For control subjects, the use of any investigated or unregistered product (drug or vaccine)

Chronic administration (over 14 days) of immunosuppressive or immunomodulatory drugs in the 6 months preceding the study

Administration of any immunoglobulin, any immunotherapy or administration of some blood products within the 3 months preceding the study or during the study period

Any medical condition or history of illness which, in the opinion of the investigator, could interfere with the evaluation of the objectives of the study

TABLE 1 Exclusion criteria to this study

3.3 Clinical characteristics of subjects with AD

Of the ten samples from subjects with AD, we had to eliminate one of them, one sample having been collected much earlier than the others and likely to skew the results; we kept nine samples of each group. Main clinical characteristics of the AD subjects are listed in Table 2, chapter 4.1

Among the nine Alzheimer's subjects selected in the study, four were in the dementia stage and five in the MCI stage. Five subjects had AD without vascular component and four had a mixed, neurodegenerative, and vascular, disease. Their age was between fifty-eight and eighty-two years old with a mean age at seventy-two years old. Recent neuropsychological screening tests (less than one year) were documented and all Alzheimer subjects had a pathological score (MoCA < 26/30 and MMSE < 24/30). Neuropsychological screening test score were all converted to MoCA scores, and scores ranged from 9/30 to 25/30. Six subjects benefited from a pro-cognitive treatment with donepezil or rivastigmine during the time of inclusion. Five subjects underwent lumbar puncture with quantification of Aβ42, t-tau and p-tau levels in the three years prior to inclusion. CSF analysis were performed at the Clinical Chemistry Laboratory of the CHUV, Lausanne, Switzerland. Of these five subjects, four had pathological Aß values (A β < 600 ng/l) in the CSF and the last one had a A β 42 value of 743.70 ng/l. However, this subject had pathological t-tau and p-tau values (t-tau > 300 ng/l, p-tau >60 ng/l), which means that all five subjects who underwent lumbar puncture had a pathological CSF compatible with AD.

3.4 APPPS1 mice

We also displayed the results of the detection and quantification of Abeta amyloid present in the feces of four transgenic mice with robust cerebral Abeta amyloidosis, i.e. APPPS1 mice. These transgenic animals co-express the KM670/671NL Swedish mutation of human APP and the L166P mutation of human PSEN1 under the control of the Thy-1 promoter. These two genes are involved in human genetic forms of AD. They show age-dependent deposition of A β peptide, starting at six weeks of age in the

cortex and 3-4 months of age in the hippocampus, with cognitive impairment in older, 8 month-old animals [39]. It has already been demonstrated that the amyloid peptide can be detected in the stool of those transgenic mice (Bolmont et al., in preparation). Animal studies were conducted according to regulations of the office fédérale de la sécurité alimentaire et des affaires vétérinaires, Swiss confederation.

3.5 Samples analysis

The eighteen stool samples collected were stored at Stemedica International Laboratory (Lausanne) at -80°C until required for analysis. Samples were anonymized before they arrived at the laboratory. The four stool samples of APPPS1 mice were harvested and stored directly in the laboratory. Measurements were all performed at Stemedica International Laboratory. First step consisted of peptide extraction of amyloid A β 42 from stool samples (see chapter 3.5.1) followed by measurement of A β 42 concentration by a sandwich ELISA technique (see chapter 3.5.2). We decided to quantify only the A β 42 peptide, this one being the most fibrillogenic and best correlated to AD pathology.

Protein extraction

In order to improve the quality of amyloid peptides detection, we intended to obtain the optimum quality in protein extract. To date, there is no commercially available kit for protein extraction in feces in view of amyloid analyses. The crucial point remains the quality of the protein extract. Stools are composed of 20% undigested material, 80% intestinal bacteria and a large amount of lipids. Therefore, we have developed a new method to preserve, precipitate and clean the fecal protein extract. Stool samples were suspended in phosphate buffered saline (PBS) buffer supplemented with sodium azide and protease inhibitors cocktail. Samples were then chilled on ice, centrifuged and supernatant was transferred to clean tubes. Supernatants were then precipitated with trichloroacetic acid (TCA) and the protein pellet was washed in ice-cold acetone, dried, and resuspended in a manufacturer-supplied diluent and in PBS.

Enzyme-linked immunosorbent assay (ELISA)

The principle of the ELISA is the adsorption of a substance on plastic and its revelation using an antibody coupled to an enzyme. Several forms of ELISA exist, but for peptide detection, ELISA sandwich technique is appropriate. Plates with different wells are incubated with solutions containing the specific antibody (here the anti-A β antibody) to the desired antigen (here the A β peptide). This antibody, fixed at the bottom of the wells, is the capture antibody. The sample is then added, and the antigens bind to the capture antibodies. A solution containing revelation antibodies, antibodies that are specific for the desired antigen is then added. The revealing antibody is coupled to an enzyme, usually a peroxidase, that generates a chemical reaction that produces a

stain. The plate is then inserted into an ELISA reader, a reader measuring the absorbance of the emitted color which is then converted into concentration.

Here, A β 42 measurements were performed using a V-PLEXTM A β Peptide Panel 1 (6E10) K15200E-1 Kit from Meso Scale Discovery. This assay uses peptide-specific capture antibodies with the n-terminal region of A β 42 and 6E10 clone as detection antibody. 6E10 clone is an antibody reactive to amino acid residue 1-16 of A β . Lower limit of detection (LLOD) is defined at 0.368 ng/l and lower limit of quantification (LLOQ) is defined at 3.13 ng/l. A β 42 measurements were performed according to manufacturer's instructions. A β 42 measurements in APPPS1 mice were performed using the same kit and methodology.

3.6 Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics version 24 commercial software. The statistical significance threshold was set with a p-value (p) at 0.05. Results were analyzed in two main groups; the Alzheimer group (ALZD) and the control group (CTRL). We also split ALZD group into two subgroups: dementia and MCI subjects. We then displayed results in APPPS1 mice group (APPPS). Variance equality in each group was evaluated with Levene's test. Normality of distribution in each group was evaluated with Shapiro-Wilk test. For comparison between two groups, we used the non-parametric Mann-Whitney U test. Non-parametric Kruskal-Wallis test was used for multiple groups comparison. The correlation coefficient of Spearman (r_s) was used as a correlation measure between variables.

4. Results

4.1 Fecal Aβ42 detection and levels

Using ELISA technique, $A\beta42$ peptide was readily detectable in the stools of subjects with AD but also in aged non-demented controls. Table 2 details the main clinical features of the nine subjects in ALZD group and the nine subjects in CTRL group, as well as the fecal amyloid values of these one. In the ALZD group, the mean $A\beta42$ value is 14.54 ng/l with a standard deviation of 4.06 ng/l and a standard error of 1.35 ng/l. In the CTRL group, the mean $A\beta42$ value is 10.10 ng/l with a standard deviation of 2.89 ng/l and a standard error of 0.96 ng/l. Figure 4 shows the distribution of $A\beta$ levels in those two groups. Figure 5 shows the distribution of $A\beta$ levels in ALZD subgroups (Dementia and MCI subjects) and CTRL group. Using a Shapiro-Wilk test, distribution in ALZD group (p = 0.926) and CTRL group (p = 0.565) is normal (a p value of more than 0.05 means that distribution is normal). Using a Levene's test, variance is found to be equal between the two groups (p = 0.627, a p value of more than 0.05 means that Variances are equal). However, due to the small number of subjects in our samples, we decided to use non-parametric comparison tests (Mann-Whitney and Kruskal-Wallis).

| | Stage of cognitive impairment | Age (years) | MoCA score | CSF | | | Fecal |
|---------|-------------------------------------|----------------|---------------|----------------|--------------|-----------------|-------------|
| Subject | | | | Aβ42 (ng/l) | t-Tau (ng/l) | p-Tau (ng/l) | Aβ42 (ng/l) |
| 1 | Dementia | 75 | 20 | | | | 14.5 |
| 2 | Dementia | 82 | 9 | | | | 17.6 |
| 3 | Dementia | 81 | 16 | 360.0 | 359.6 | 55.7 | 22.4 |
| 4 | Dementia | 77 | 22 | | | | 12.7 |
| 5 | MCI | 64 | 15-16 | 514.0 | 712.1 | 99.1 | 8.1 |
| 6 | MCI | 58 | 22 | 474.3 | 2268.9 | 291.9 | 15.6 |
| 7 | MCI | 65 | 17 | 414.1 | 590.8 | 71.2 | 11.0 |
| 8 | MCI | 75 | 19 | | | | 13.4 |
| 9 | MCI | 72 | 25 | 743.7 | 740.1 | 99.0 | 15.6 |
| 10 | Control | 63 | | | | | 13.7 |
| 11 | Control | 70 | | | | | 7.5 |
| 12 | Control | 72 | | | | | 9.8 |
| 13 | Control | 67 | | | | | 11.6 |
| 14 | Control | 58 | | | | | 12.5 |
| 15 | Control | 55 | | | | | 13.4 |
| 16 | Control | 67 | | | | | 7.4 |
| 17 | Control | 69 | | | | | 5.6 |
| 18 | Control | 63 | | | | | 9.4 |

TABLE 2 Clinical features and fecal A β 42 levels of the nine AD subjects and the nine control subjects included in this study

Physiological standard for biomarkers in the CSF: $A\beta 42 > 600 \text{ ng/l}$, t-Tau < 300 ng/l, p-Tau < 60 ng/l according to the Clinical Chemistry Laboratory (LCC) of the CHUV.

Comparison between ALZD and CTRL

Fecal A β 42 levels tended to be higher in Alzheimer's subjects (mean value 14.54 ng/l) compared to control subjects (mean value 10.10 ng/l). The mean difference is 4.44 ng/l, representing an increase of 43.96% in mean amyloid levels compared to controls. The AD to control ratio is 1.44. Using a Mann-Whitney U test (U = 13.5, p = 0.015), difference in means between Alzheimer and control samples is statistically significant. We can notice in the Alzheimer's group, the presence of a subject with a higher rate of A β peptide (22.40 ng/l) than the rest of the group.



Comparison between ALZD subgroups and CTRL

Inside the Alzheimer group, fecal A β 42 level tended to be higher in dementia subjects (mean value 16.80 ng/l) compared to MCI subjects (mean value 12.74 ng/l). The mean difference is 4.06 ng/l, representing an increase of 31.87% in mean amyloid levels. Using a Mann-Whitney U test (U = 5.0, p = 0.254), difference in means between dementia and MCI subjects is not statistically significant. Logically, dementia subjects had also higher fecal A β 42 levels than control subjects (mean value 16.80 ng/l and 10.10 ng/l respectively), with a difference in means using a Mann-Whitney U test (U = 2.0, p = 0.011), that is statistically significant. MCI subjects had also higher fecal A β 42 levels than control subjects had also higher fecal A β 42 levels than control subjects had also higher fecal A β 42 levels than control subjects had also higher fecal A β 42 levels than control subjects had also higher fecal A β 42 levels than control subjects had also higher fecal A β 42 levels than control subjects (U = 1.5, p = 0.156) that is not statistically significant. We also performed a non-parametric Kruskal-Wallis test (Chi-Square 6.978, p = 0.022) to compare mean A β 42 levels and results showed that difference in means in these three groups were statistically significant

In summary, statistical difference in fecal A β 42 means was found between Alzheimer subjects and control subjects and between dementia and control subjects. Other results were not statistically significant. However, because of the small number of subjects, these statistics are indicative of a trend and cannot claim any interpretation in term of significance.

Fecal A β 42 in APPPS1 mice

Detection of the peptide in the stools of APPPS1 mice had already been demonstrated in a previous preclinical investigation (Bolmont et al., in preparation). In these 4 transgenic mice, a mean A β 42 value of 27.35 ng/l was detected, with a standard deviation of 7.75 ng/l and a standard error of 3.88 ng/l.

4.2 Correlation between fecal A β 42 levels and CSF A β 42 levels / clinical impairment

Cautionary note: Due to the small number of subjects, the correlations measured below in chapter 4.2 and 4.3 are able to highlight some tendencies but are not statistically significant and therefore do not allow interpretation.

We decided to compare fecal A β 42 levels with CSF A β 42 levels and cognitive function in AD subjects. We hypothesized that the level of fecal A β 42 peptide is directly correlated with the level of cerebral A β 42 peptide, and that cerebral A β 42 is correlated to cognitive performance. Cognitive performances were assessed by the MoCA neuropsychological screening test. Correlation between fecal A β 42 levels and CSF A β 42 levels is shown in figure 6 and correlation between fecal A β 42 levels and MoCA score is shown in figure 7. Using a Shapiro-Wilk test, distribution of CSF A β 42 (p = 0.374) and MoCA score (p = 0.750) in ALZD group is normal (a p value of more than 0.05 means that distribution is normal). However, due to the small number of subjects, we decided to measure correlation with the non-parametric Spearman correlation analysis.

Of the nine Alzheimer subjects, only five of them had a lumbar puncture, reducing the sample size to five subjects for this analysis. Between fecal and CSF A β 42 levels (n = 5), we measured a Spearman correlation coefficient of $r_s = -0.410$ (p = 0.493), showing a moderate and inverse correlation between those two variables. Unfortunately, the relationship was not statistically significant. Between fecal A β 42 levels and MoCA score (n = 9), we measured a Spearman correlation coefficient of $r_s = -0.04$ (p = 0.991) showing no correlation between those two variables. Again, the relationship was not statistically significant.

4.3 Correlation between fecal Aβ42 levels and age

We decided to compare fecal A β 42 levels with age in AD subjects and controls. Given the fact that age is associated with amyloidogenesis, we hypothesized that level of fecal A β 42 is positively correlated with age. Correlation between fecal A β 42 levels and age in Alzheimer subjects is shown in figure 8 and in control subjects in figure 9. Using a Shapiro-Wilk test, distribution of age in ALZD group (p = 0.516) and in CTRL group (p = 0.568) is normal (a p value of more than 0.05 means that distribution is normal). However, due to the small number of subjects, we decided to measure correlation with the non-parametric Spearman correlation analysis.

In Alzheimer group, we measured a Spearman correlation coefficient of $r_s = 0.504$ (p = 0.166), showing a strong and positive correlation between fecal A β 42 levels and age. Unfortunately, the relationship was not statistically significant. In control group, we surprisingly measured a Spearman correlation coefficient of -0.630 (p = 0.069), showing a strong and inverse correlation between fecal A β 42 and age. Again, the relationship was not statistically significant.





5. Discussion

To date, diagnosis of AD remains mainly clinical. The arrival in recent decades of biomarkers of the disease remains a considerable advance, unfortunately, the only biochemical biomarker available is found in the CSF and therefore requires invasive procedure to harvest it. No reliable peripheral biomarkers exist today, the ones having been recently evaluated, in the blood mainly, did not show clinical relevance. Recent findings on the metabolism and physiopathology of the A β peptide have shown that the peptide is transported into blood system and peripheral organs, and that it is probably eliminated from the periphery partly by bile excretion in the stools. Given the urge to develop new peripheral biomarkers of AD, the research for A β peptide in the stool seems to be a promising avenue. Until today, no biomarker research for AD has been performed in the stool.

In this study, we managed to detect the A β 42 peptide in the stools of subjects with AD and in aged non-demented controls using ELISA. We only detected and quantified this

peptide alone but, in further studies, we could normalize the quantification by measuring its ratio to total fecal protein content. To date, there is no bona fide commercially available kit to extract Abeta protein from fecal matter. Effort should be made in developing a better solubilization buffer for the waste material and therefore increase fecal peptide detection sensitivity. Currently, there is no "gold standard" test for the detection and quantification of amyloid peptide in stools. For example, the gold standard of the FIT (Fecal Immunochemical Test) for colorectal cancer screening is colonoscopy and the gold standard for measurement of fecal calprotectin for the diagnosis of IBD (inflammatory bowel diseases) is the detection of fecal leucocytes labeled with indium-111 and colonoscopy [40,41]. The performances of this ELISA test will have to be compared and validated against a reference method. We could also compare the ELISA technique to a traditional western blot technique, which could provide a precise and semi-quantitative fecal peptide detection. It would also be important to evaluate the reproducibility of the measure of fecal amyloid in the same subject. Indeed, it is possible that the level of the fecal Aß peptide is influenced by certain factors external to AD such as diet, medication, presence of variable intestinal inflammation, hepatic function, renal function [18] etc.

Fecal A β 42 levels were significantly higher in Alzheimer's subjects (mean value 14.54 ng/l) compared to control subjects (mean value 10.10 ng/l, AD to control ratio 1.44, p = 0.015). Inside the Alzheimer group, fecal A β 42 level tended to be higher in dementia subjects (mean value 16.8 ng/l) compared to MCI subjects (mean value 12.74 ng/l, p = 0.254), in dementia subjects compared to control subjects (p = 0.011) and in MCI subjects compared to control subjects (p = 0.156). Much, if not all results were found to be not statistically significant, mainly due to the low number of subjects. But we can notice that the more the cognitive disorder is advanced, the more the difference in mean A β 42 levels with controls is significant (dementia vs control p = 0.011, Alzheimer vs control p = 0.015). In summary, we showed that A β 42 peptide levels are higher in AD subjects than in non-demented subjects, and it seems that the peptide level is directly correlated to the severity of the disease, i.e. the severity of the cognitive and functional impairment. However, larger studies need to be conducted to confirm theses tendencies and to determine if a cut-off, a value above which the test is considered pathological, can be fixed.

A β 42 levels were higher in the stool of APPPS1 mice (mean value 27.35 ng/l) compared to human samples (mean value 12.32 ng/l), although rates are not comparable. In APPPS1 mice, APP and PSEN1 mutation is directly associated with overexpression of the A β peptide, thereby generating significantly higher levels of amyloid peptide than in the sporadic forms of AD. In our Alzheimer sample, we selected a subject that suffers from a genetic form of AD linked to APP mutation. In this subject, an A β 42 level of 15.6 ng/l was detected, this level being not significantly higher than that of the rest of Alzheimer subjects. We can therefore not confirm that genetic form of AD in human is associated with higher A β 42 stool level than in sporadic disease. Again, larger scale studies need to be performed to determine whether subjects with genetic form of AD have higher level of fecal A β 42 than those with sporadic AD.

We also tried to measure the correlation between fecal A β 42 levels, CSF A β 42 and cognitive performances. Unfortunately, every correlation measured here were not

statistically significant, due to the low number of subjects. However, we can hypothesize that fecal Aβ42 is correlated with progression of the disease, characterized by the stage of cognitive impairment (dementia or MCI), the MoCA score and CSF Aβ42 levels. Of course, larger scale studies need to be done to determine if this hypothesis is correct, namely if there is an inverse correlation between fecal amyloid and CSF amyloid, and between fecal amyloid and cognitive performances. A big difficulty is the accurate assessment of the cognitive deficit of each person with AD. Tests such as MoCA and MMSE are only screening tools and are therefore not a reliable assessment of the patient's cognitive and functional impairment. Results of these screening tests cannot be reliably correlated with biomarkers of the disease. A deeper neuropsychological and functional assessment should be performed for correlation analysis in clinical research.

We finally tried to measure correlation between age and fecal Aβ42 levels in Alzheimer group and control group. Since physiological aging is associated with the process of amyloidogenesis, we expected to find a positive correlation between age and fecal amyloid levels. Again, all correlation measured were not statistically significant, but we can reasonably imagine that in larger studies, a positive correlation should be detected between age and fecal amyloid levels, regardless of the sample. Measuring the correlation between fecal amyloid levels and other disease variable, such as age, cognitive performance and CSF amyloid levels is difficult, each variable influencing the others. In summary, all the correlation studies that we have performed have returned not significant, mainly due to the low number of subjects. We are just able to highlight some tendencies and larger scale studies need to be conducted to confirm them.

6. Conclusion and outlook

In this prospective clinical study, we demonstrated the feasibility of detecting A β 42 peptide human stools using a standard ELISA technique. Quantification of the peptide in the stools showed higher levels in Alzheimer subjects compared to control individuals without significant brain amyloidosis. We also provided some evidence of a possible correlation between the levels of fecal A β 42 peptide and the progression of the disease as characterized by the stage of cognitive impairment (MCI or dementia), the MoCA score and CSF A β 42 levels.

However, we must mention several limitations of this study. First, we probably had a selection bias of our subjects. Alzheimer subjects and control subjects were not selected in the same way, Alzheimer subjects having been selected according to rigorous criteria unlike control subjects. Another problem is the diagnosis of AD: the subjects have a probable AD and it is therefore possible that the diagnosis is false, for example confused with a vascular dementia or another neurodegenerative dementia. We also did not verify the absence of significant cerebral amyloidosis in non-demented control subjects; it is consequently possible that non-demented subjects suffer from cerebral amyloidosis potentially compatible with preclinical AD which may skew the results. The last limitation concerning our samples and probably the most important is

their size; we used a small number of participant and therefore, we only highlighted some tendencies that will have to be confirmed by larger studies.

Nevertheless, the use of stool as a tool for diagnosis and screening of Alzheimer's disease is a serious track not to neglect. It could provide a noninvasive diagnosis or screening of AD, as it is already the case with the FIT for colorectal cancer screening or the fecal calprotectin quantification for IBD diagnosis. Yet, we have noticed a certain difficulty concerning the stool harvest process in patients with cognitive disorders. Indeed, the harvest process requires a complete comprehension and participation of the person and does not seem adapted to the presence of major cognitive disorders. We could however offer this test to patients suffering from AD in an early stage (MCI stage). We could as well imagine delegating this gesture to caregivers and performing these stool collections in institutionalized subjects in a medico-social institution. In addition to the detection of the AB peptide, we could imagine detecting other biomarkers in the stool such as p-tau or t-tau peptide. Recent studies appear to involve the gut microbiota in the physiopathology of AD [42,43]. Sequencing of fecal microbiota could therefore also be included in the list of potential new biomarkers of AD (Bolmont et al., in preparation). We could also perform these fecal peptide detections in other neurodegenerative diseases, such as α-synuclein detection for Parkinson's disease or Lewy Body disease.

7. Bibliography

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