Rapid improvement of canine cognitive dysfunction with immunotherapy designed for Alzheimer's disease

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Running headline: Aβ immunotherapy ameliorates dog CDS
ABSTRACT:
Immunotherapy against amyloid-beta (Aβ) may improve rodent cognitive function by reducing amyloid neuropathology and is being validated in clinical trials with positive preliminary results. However, for a complete understanding of the direct and long-term immunization responses in the aged patient, and also to avoid significant side effects, several key aspects remain to be clarified. Thus, to investigate brain Aβ clearance and Th2 responses in the elderly, and the reverse inflammatory events not found in the immunized rodent, better Alzheimer's disease (AD) models are required. In the aged familiar canine with a cognitive dysfunction syndrome (CDS) we describe the rapid effectiveness and the full safety profile of a new active vaccine candidate for human AD prevention and treatment. In these aged animals, besides a weak immune system, the antibody response activated a coordinated central and peripheral Aβ clearance, that rapidly improved their cognitive function in absence of any side effects. Our results also confirm the interest to use familiar dogs to develop innovative and reliable therapies for AD.

Keywords
INTRODUCTION

Alzheimer’s disease is characterized by the presence of intracellular neurofibrillary tangles and extracellular Aβ plaques, with Aβ monomers and oligomers resulting from the amyloid precursor protein (APP) processing [1]. In the AD amyloid hypothesis, the extracellular accumulation of soluble Aβ peptide leads to CNS formation of soluble oligomers, fibrils and amyloid plaques, and the distribution between brain and blood compartments, reflects the imbalance between Aβ production and clearance [2]. The Aβ fibril formation is currently considered a dynamic and reversible process, as Aβ fibrils are continuously dissolving and reforming [3]. Because AD stage severity correlates more with soluble Aβ brain levels than with Aβ plaque abundance [2,4,5], the amyloid monomers and oligomers are considered to be more toxic [1,6]. Blockade of the AD course at early phases through different immunological anti-Aβ strategies to interfere the amyloid peptide homeostasis have given promising results. For example, active immunization targeting Aβ peptide in AD Tg mice has demonstrated striking reductions in Aβ deposition and reduction of behavioral impairments [7–9], and the same was observed in human beings [10]. However, significant questions remain to be answered for a full understanding of the direct and long-term immunization responses, and to avoid significant side effects in trials conducted with human patients. Thus, the development of new active vaccines inducing both Aβ clearance and only Th2 responses in good AD models remain key issues to be solved [11,12]. Dog with cognitive dysfunction syndrome (CDS) is considered a suitable model to address these innovative therapies [13], because it spontaneously expresses several markers associated with the underlying pathology of sporadic AD. Control aged dog (over 7-8 years) is also considered quite unique to elucidate the amyloid hypothesis, since its CNS Aβ peptide accumulation is responsible for neuronal dysfunction and deregulation of the same specific cortical subsets of interneurons described in the aged
human [14–17]. In addition, these aged animals also bring multiple druggable targets for the identification of novel disease-modifying agents to interfere natural Aβ production, aggregation, clearance or the associated neurotoxic effects [18,19].

Compared to the aged housed dog, some familiar dogs develop geriatric behavioral changes that give rise to a specific CDS, also known as canine counterpart of senile dementia of the Alzheimer's type. Because of their many common hallmarks, CDS is presently considered a model of choice for Mild Cognitive Impairment (MCI) and AD research [15,20,21], and, in particular, for the development of anti-Aβ immunotherapy [13,22,23]. Diagnosed of graded cognitive dysfunctions by practicing neurology veterinarians, these animals are systematically and easily classified into well-characterized homogenous groups with no variation due to their breed (including sex, size and genetic background) or familiar life conditions [24–26]. In this study, we took advantage of this familiar model to investigate the effectiveness and safety of new active amyloid vaccines. This study is aimed at increasing our understanding of how immunomodulation supports CNS Aβ clearance, how it relates to cognitive improvement, and how it supports a safe and effective new AD immunotherapy.
MATERIAL AND METHODS

Vaccine preparation
Three different fibrillar Aβ vaccines (v1, v2 and v3) were initially prepared with 100µg of either Aβ40 peptide (>95% purity; Genscript, NJ, USA) (v1), 100µg Aβx-40 peptide KLH conjugated (>95% purity; Anaspect, CA, USA) (v2), or 100µg of 2/3 Aβ40 combined with 1/3 of the Aβx-40 peptide KLH conjugated (v3). Aβ40 and Aβx-40 peptides were initially dissolved with dimethyl sulfoxide (DMSO) (1mg/ml), aliquoted and stored at -20ºC. Once thawed, each solution was mixed with 1mg of alum hydroxide suspension (Sigma, Spain). After overnight (ON) incubation at 4ºC, a saline solution was added up to a volume of 1.5ml, previous to the subcutaneous (s.c.) immunization of the dog. Placebo animals only received a mixture of saline solution + alum hydroxide suspension.

Human samples
Brain tissue from AD patients, stage II according to the Braak & Braak scale [27] was obtained from the Neurological Tissue Bank of the Universitat de Barcelona, Barcelona, Spain. 5µm paraffin embedded slices from hippocampus and cerebral cortex were obtained for immunohistochemistry experiments.

Animals
To select the vaccine of major interest, 6 adult female New Zealand White rabbits weighing between 1.5-2 kg at the beginning of the study were immunized with one of the 3 vaccines following the protocol selected for dogs. They were kept on a 12h light/12 h dark cycle and housed with free access to food and water.
The safety and immunization studies were performed in 36 dogs (12 housed beagle dogs + 24 familiar dogs). The 12 male housed beagle dogs from Harlan laboratories (Gannat, France), with an age ranging between 2 and 8 years at the beginning of the study, were housed in groups of three in 1.85m wide x 3m large buildings, and were
daily fed with 340gr of Hill's canine adult food. The 24 familiar dogs of different breeds, sex and age were classified into control and dogs with graded CDS (Table 1) by a veterinarian neurologist.

All the animals were treated according to the European legislation on animal handling and experimentation (86/609/EU) and, with the formal agreement of the owners of the familiar dogs. Procedures were approved by the Ethics Committee of the University of Barcelona, Barcelona, Spain. All efforts were made to minimize animal suffering and to use no more than the number of animals needed to obtain reliable scientific data.

**Cognitive evaluation**

The cognitive status of all 24 familiar dogs was evaluated. A validated cognitive test of 9 items adapted from the Mini-Mental State Examination (MMSE) and the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) for AD was used in collaboration with the pet owners (Table 2) [13,21]. Based on the presence of established housetraining habits, disorientation in familiar surroundings, decreased activity, playfulness, vitality, decreased interaction with the owner, and modifications of the sleep/awake cycle, dogs were divided into two groups: Old Control dogs (OC) and CDS dogs. Depending on the dysfunction grade, the CDS group was subdivided into a group with light cognitive deficit (LCD) or severe cognitive deficit (SCD).

**Screening study**

For the screening study all the 36 dogs were classified following their age and cognitive status in Adult Control dogs (AC), OC and LCD or SCD (Table 1).

For each dog, a biochemical and hematological profile was regularly performed that also included Aβ levels in blood and Cerebrospinal Fluid (CSF). The albumin coefficient reflecting the Blood-Brain-Barrier (BBB) integrity [28] was also calculated. All animals were also monitored for adverse reactions and the vaccine puncture zone was periodically revised.
Immunization study

Following the recommendation of the Ethics Committee, the use of healthy adult and old familiar dogs as control animals for the immunization study had to be avoided as the following 21 dogs were considered sufficient to obtain reliable data: 9 housed beagle dogs as control group and 12 CDS familiar dogs, with the acceptation of their owners, as the Case group (Table 1).

The study was performed over a period of 131 days, with 6 s.c. injections administered during a period of 51 days in the back of the neck (Fig. 1). 6 housed beagle dogs were immunized with the v3 vaccine (Immunized AC) and 9 dogs CDS dogs were immunized (Immunized CDS). The remaining 3 housed adult beagles and 3 familiar dogs were included in the Placebo group (Table 1). Cognitive follow-up of all CDS animals was performed on a double blind basis at days 31, 51 and 131, by the veterinarian and the pet’s owner, and blood and CSF parameters were regularly evaluated (Fig. 1).

Blood and CSF extraction

Serum and plasma were obtained after 5ml blood extraction from the animal jugular vein every 10 days until day 71, and then at day 131. Once collected in tubes (BD, NJ, USA) containing EDTA for plasma or clotting gel for serum, blood was centrifuged at 3000rpm during 15 minutes, aliquoted and kept at -80°C until use. CSF was collected under 10mg/kg of sodium thiopental (Thiobarbital, Braun Medical S.A., Spain) intravenous (i.v.) anesthesia. Then, dogs were intubated, and oxygen administered following induction. Monitoring devices were used to control heart rate, blood pressure and blood oxygenation. The dog’s coat was clipped and prepared for an aseptic procedure, and CSF collected (1ml/5kg) by cisternal puncture into a sterile glass tube. Afterwards, it was divided into 0.5ml aliquots and quickly stored at -80°C until use.
Biochemical and hematological analysis

Biochemical parameters were analyzed by spectrophotometry (DIRUI CS-800, RAL, Barcelona, Spain) and hematological parameters were measured by laminar flow impedance (Procyte DX, Barcelona, Spain) (Table 3). The albumin quotient (Qalb) was calculated as the quotient between the albumin CSF (mg/L) and blood (g/L) concentration. CSF albumin was quantified by immunonephelometry (BN2, Siemens, Marburg, Germany) and serum albumin with the colorimetric bromocresol method (ADVIA 1800, Siemens diagnostic, Marburg, Germany).

Anti-Aβ IgG titration

For the rabbit anti-Aβ IgG titration, a Maxisorb 96 well plate (Nunc, Langenselbold, Germany) was coated ON at 4°C with 100µl of Aβ40 diluted at 2µg/ml in NaHCO₃. After a wash with 200µl of Phosphate buffer saline 100mM, pH 7.4 + 0.05% Tween (PBST), the plate was blocked during 2h at room temperature (RT) with PBS-bovine serum albumin (BSA) 5%. 100µl of rabbit serum from the different time points was diluted 1/100 with PBS-BSA 5% and added to the wells after 3 washes during 2h at RT. In 3 wells, only diluted PBS was added as a background reference. After 4 washes the biotin anti-rabbit IgG (Sigma Aldrich, MO, USA) diluted at 1/20000 was added for 1h at RT. Finally, and after 5 washes, extrAvidin peroxidase 1/200 was added for 30min and developed with 100µl of 3,3',5,5' tetramethylbenzidine (TMB) during 5 min. The reaction was stopped with 100µl H₂SO₄ 100mM, and the optical density (OD) registered at 450nm (Tecan, Männedorf, Switzerland).

For the dog serum and CSF anti-Aβ IgG titration, a Maxisorb 96 well plate was also used and coated ON at 4°C with 100µl of Aβ₄₀ diluted at 2µg/ml in NaHCO₃. The plate was then washed with 200µl of Tris buffer saline 50mM + 0.05% Tween pH 7.4 (TTBS) and blocked with TBS-BSA 5% for 2h at RT. After washing, 100µl of each diluted serum or CSF sample was added in triplicate and incubated for 2h at RT. Serum samples were diluted at 1/200 with TBS-BSA 5% and CSF samples at 1/2 with TBS.
Samples from each dog were included in the same plate and 3 wells added with TBS-BSA 5% were also used for background. After 5 washes, each well was incubated for 30min at RT with a biotin rabbit anti-dog IgG (Lifespan bioscience, Derio, Spain) diluted with TBA-BSA 5% at 1/40000. After 6 washes, streptavidin diluted 1/4000 (Invitrogen, Madrid, Spain) was added and the ELISA kit amplification system (Invitrogen, Madrid, Spain) used for 2 min. Reaction was stopped with H2SO4 0.3M and OD registered at 492nm. Data from IgG anti-Aβ titration of the different plates were normalized versus background.

Quantification of Aβ_{40} and Aβ_{42} in plasma and CSF

A specific ELISA kit (IBL international, Hamburg, Germany) was used for the detection of the total Aβ_{40} and Aβ_{42} in plasma and CSF. Following the manufacturer's guidelines, standard curves, controls and calibration were performed. Samples were also diluted 1:3 for the measurement of CSF Aβ_42, 1:25 for CSF Aβ_{40} and 1:5 for plasma Aβ_{40}. After 7 washes with PBS 100mM, pH7.4 containing 0.05% Tween-1%BSA, Aβ species were detected with anti- Aβ antibodies and measured at 450nm.

Immunohistochemistry

Immunohistochemistry was carried out on paraffin sections of brain tissues from stage II AD patients according to the Braak & Braak scale. Purified rabbit anti-Aβ antibodies from serum of v1, v2 and v3 immunized rabbits or purified dog anti-Aβ from immunized AC and immunized CDS dogs or commercial anti-Aβ antibodies (Dako, Glostrup, Denmark) were used as a primary antibody to detect Aβ plaques. Slices were deparaffined and pre-treated in formic acid for 2min. After endogenous peroxidase inhibition for 30min, slices were incubated with PBS 100mM, pH 7.4, containing 0.5% triton-X100-Normal goat serum 5%-BSA 5% as a blocking agent for 2h at RT. Purified primary antibodies were diluted with immunobuffer (PBS-5%-triton-X100-1%NGS-1%BSA) at a correct concentration, or commercial anti-Aβ antibody diluted at 1/100 were added an incubated ON at 4°C. After 3 washes with PBS, samples were
incubated with 1/4000 diluted biotin rabbit anti-dog IgG (Lifespan bioscience, Derio, Spain) or 1/500 biotin mouse rabbit IgG for 2h at RT. Finally, after 3 washes, slices were incubated for 1h with ExtrAvidin peroxidase (1/250) and developed with 0.03% Diaminobenzidine (DAB) 0.006% H₂O₂ in PBS. Sections were dehydrated and mounted in DPX. Plaques were visualized by optical microscopy (Olympus America Inc., NY, USA) and micrographs were taken at 5x and 40x.

**Statistical analysis**

Kurtosis and skewness moments were calculated to verify the normal distribution of data. One-way ANOVA was performed to compare CSF and serum parameters between animal groups. To analyze differences in CSF and serum parameters during the treatment, two-way ANOVA was performed with two factors: Time with 5 levels (day 0, day 31, day 51, day 71 and day 131) of serum parameters, or with 3 levels (day 0, day 51 and day 131) of CSF parameters, and Treatment (Non-treated, Placebo and Treated). When significant two-way interactions were observed, individual comparisons were performed using one-way ANOVAs followed by LSD post-hoc test. To quantify the association between two variables, a Pearson correlation was performed. When normality was not achieved, the values of all groups were compared using Non-parametric analysis. In these cases, Kruskal-Wallis (KW) test followed by Mann-Whitney test (MW) were used to compare dependent variable groups, and Sperman correlation to quantify the association between two variables. In all cases, p<0.05 was considered as significant. Results are expressed as a mean ± SEM. All analyses were performed with the programs SPSS 15 (IBM España, Madrid, Spain) and STATGRAPHICS (STSC Inc, Rockville, MD, USA).
RESULTS

The v3 vaccine induces the highest antibody titration in rabbit

To select the vaccine of major interest, an immunization study with v1, v2 and v3 was initially conducted in rabbits. To this end, 6 animals (n=2/group) were immunized with one vaccine following the immunization protocol selected for dogs. Blood samples were also extracted at the same selected time points until day 71, and anti-Aβ IgG measured. All three vaccines induced immunization, however the v3 composition gave the highest anti-Aβ IgG titration at day 71 (Fig. 3A). No side effect was detected in any animal.

Dogs with light cognitive deficits have increased levels of plasma protein, platelets and Aβ levels

When the 24 familiar dogs were evaluated at the beginning of the study (day 0) with our cognitive test, CDS was identified in 14 animals that presented light cognitive deficits (LCD, n=10) or severe cognitive deficits (SCD, n=4) (Table 2). The remaining 10 animals classified as control were distributed with the 12 housed beagle dogs between the Adult Control group (AC, < 8 years, n=14) and the Old Control group (OC, n=8).

During the whole study, all 16 biochemical and hematological parameters were found in the normal range in all animals. However, in the CDS group plasma protein levels and platelets was always higher and reticulocyte counts lower (p=0.004, 0.001 and 0.002, respectively) (Data not shown).

LCD dogs presented the highest Aβ40 plasma level (p=0.029) (Fig. 2A), and in all groups Aβ42 was lower than the plasma detection limit. In CSF, Aβ40 levels remained stable with aging and cognitive dysfunction, while Aβ42 levels only increased in the LCD dogs (p=0.018) (Fig. 2B,C). The high variability of their CSF Aβ42 values were strongly reduced once subdivided into: LCD1 dogs characterized by CSF Aβ40 and Aβ42 levels
similar to those of the Control and SCD groups and highest Aβ\textsubscript{40} plasma values (p=0.009) (Fig. 2A, B, C); and LCD2 dogs with the highest CSF Aβ\textsubscript{42} levels (p=0.018), and Aβ\textsubscript{40} plasma and CSF levels similar to those of the Control and SCD groups (Fig. 2C). When this new classification was applied to the plasma protein levels and platelet and reticulocyte counts, differences were observed in the LCD1 group (p=0.009, 0.024 and 0.010, respectively) that did not correlate with Aβ\textsubscript{40} or Aβ\textsubscript{42} plasma and CSF values. Finally, only in controls Qalb positively correlated with age (R\textsuperscript{2}= 0.56, p=0.0008) (Fig. 2D).

Cognitive improvement of v3 immunized CDS dogs is accompanied by an increased plasma/CSF Aβ ratio

The cognitive test was performed at days 0, 31, 51 and 131 in all dogs included in the v3 immunization study. At day 31 the immunized CDS dogs presented a 30% lower score (p=0.027) (Fig. 3B) that was maintained until day 51. At this day, a greater reduction of the score was observed in SCD dogs (45%, p=0.010) than in LCD ones (25%, p=0.043). A tendency to decrease was found in placebo animals, probably due to a closer and more frequent owner and veterinarian care. Due to a failing of several owner visits at the end of the study, only two immunized dogs could be evaluated until day 131. Compared to days 51, 31 and 0, both animals gave a lower score (Score values=11 and 9).

To evaluate the molecular response to v3 immunization, all 16 hematological and biochemical blood parameters, as well as the blood and CSF Aβ and anti-Aβ\textsubscript{40} IgG levels, were measured during the whole immunization period.

Only the Alkaline Phosphatase (AP) value was not within the normal range in some familiar dogs (Table 3). Higher AP levels at days 71 and 130 (p=0.023 and 0.049, respectively) were detected in immunized CDS dogs compared to the placebo group, and were probably related to the irregular familiar feeding habits.
Qalb ratio remained similar in the AC and CDS groups during the whole study (Data not shown).

In the v3 immunized AC dogs, Aβ_{40} plasma levels presented a tendency to increase until day 41. The same tendency, but with a larger variability, was observed until day 51 in the v3 immunized CDS group (Fig. 4A). When compared with placebo, immunized AC dogs presented a similar Aβ_{40} CSF level between day 0 and 131, while Aβ_{42} value peaked at day 131 (p=0.034). In the immunized CDS group, Aβ_{40} CSF levels presented a tendency to decrease (Fig. 4B), and Aβ_{42} levels were lower at day 51 (p=0.049) (Fig. 4C). When the ratio between plasma and CSF Aβ was obtained, a major tendency to increase was found in the immunized CDS dogs compared to placebo and AC immunized animals (Fig. 4D). When the analysis was performed with the immunized LCD1, the increasing values reached significant differences between LCD1 immunized dogs and Placebo and AC animals at day 51 (p=0.0211) (Fig. 4H).

Significant plasma and CSF changes of the anti-Aβ_{40} IgG levels were observed in all v3 immunized dogs during the whole follow-up. Thus, in the immunized AC group, the anti-Aβ_{40} IgG serum levels reached a peak at day 31 and remained increased until day 51 when compared to its pre-immune (p=0.0042) and Placebo group values (p=0.0079); at day 131 it returned to baseline value. Anti-Aβ_{40} IgG serum levels peaked at day 71 in immunized CDS dogs, higher that their pre-immune (p=0.0078) and the Placebo group values (p=0.0022) (Fig. 4E), being back to baseline at day 131. CSF Anti-Aβ_{40} IgG of immunized AC dogs were increased at day 51 compared to pre-immune (p=0.0042) and placebo values (p=0.0078). CDS animals showed a similar tendency, but with lower levels (Fig. 4F). Both AC and CDS groups presented a positive correlation between serum and CSF anti-Aβ_{40} IgG levels (r²=0.53, p=0.0013) (Fig. 4I). However, only in the immunized CDS group and during the whole study, the anti-Aβ_{40} IgG and Aβ_{40} plasma ratio was 1/25, with a positive and linear correlation during the whole study (r²=0.362, p=0.0049) (Fig. 4G).
Anti-\(\text{A}\beta 40\) IgG from v1, v2 or v3 immunized rabbits and v3 immunized dogs recognize human amyloid plaques

In cerebral cortex of human with AD, immunohistochemistry with plasma anti-\(\text{A}\beta\) IgG isolated from the different immunized rabbits and dogs specifically stained amyloid plaques but not vessels. Purified antibodies from serum of v1 and v3 immunized rabbits presented a strong immunodetection of diffuse and core amyloid plaques, but the ones from v2 immunized rabbits only detected diffuse plaques (Fig. 5B, C, D). Purified dog anti-\(\text{A}\beta\) IgG presented a stronger staining of diffuse plaques than of core plaques (Fig. 5F, G).

DISCUSSION

Our data reinforce the CDS dog as a good natural AD model and fosters immunotherapy for \(\text{A}\beta\) pathologies, like canine CDS or human AD. Amyloid pathology associated with canine cognitive dysfunction presents a positive correlation between plaque maturation and degree of cognitive dysfunction [24,29]. In this study, CDS familiar dogs were classified based on their cognitive dysfunction degree and age in order to identify specific biomarkers of amyloid homeostasis of help for AD.

Firstly, the higher values of plasmatic proteins and lower reticulocyte number found in the LCD1 group may reflect the oxidative damage and inflammatory processes involved in the first stages of the disease. Initial amyloid deposition, related to oxidative stress and inflammation [17,30], might then be associated with alterations of hematological parameters present in aging and oxidative damage [31]. Secondly, we also found higher plasma levels of \(\text{A}\beta_{40}\) in the LCD1 group, which would indicate a relationship between plasma \(\text{A}\beta_{40}\) and cognitive dysfunction. This hypothesis, approached in rodents and dogs with conflicting results [32–34], is difficult to validate due to the \(\text{A}\beta\) hydrophobic properties and its important binding to plasma proteins, such as albumin, that cause analytical interferences and variability.
Similarly, although no relationship has been established between Aβ plasma levels and BBB permeability, several studies in human beings, dogs and rodents describe an increased BBB permeability with aging [35,36]. However, besides the association between BBB permeability and inflammatory diseases [37–40], no correlation has been established between BBB permeability and cognitive dysfunction [39,41]. Our results evidence that BBB permeability is only increased in aging healthy dogs, and remains stable in CDS dogs, similar to young matched animals, reflecting a lack of BBB alteration associated with cognitive dysfunction.

In fact, our findings reinforce the familiar dog to help decipher the early events of the diffuse Aβ plaque maturation and explain its relationship with human cognitive dysfunction [13]. For example, together with differences in platelet levels in the LCD1 group, a comparison between these dogs and the human MCI [42], specifically with amnesic MCI, in which the disease progress to AD might bring new advances.

Finally, we found no correlation between CSF Aβ42 and cognitive dysfunction, and only higher Aβ42 levels in the LCD group that could be used as a marker of early CDS. CSF Aβ42 level, a biomarker for AD diagnosis and monitoring correlates negatively with total Aβ load in the brain, and also with cognitive dysfunction stage [43–45]. On the other hand, our CSF results are similar to those described recently in control housed beagles showing that, between the age of 4 and 16, CSF Aβ40 remains constant and Aβ42 presents a slight decrease [14]. However, the lack of data regarding Aβ42 and Aβ40 plasma variation makes difficult a further amyloid homeostasis explanation. Different dynamic [3] and Aβ aggregation progression processes [46–49] may explain these differences with the studies in human beings.

Despite the differences between some CDS and AD biomarkers, we can establish a relationship between canine cognitive dysfunction and age-related amyloid biomarkers. The dog cognitive improvement associated with changes in several biomarkers verifies the potential of our Aβ vaccine for CDS treatment. Our findings also confirm the interest of immunotherapy to stimulate Aβ peptide clearance across the BBB for a rapid and
safe cognitive benefit in CDS dogs. Thus, as early as 30 days after initial v3 immunization, plasma Aβ40 was increased and both CSF Aβ40 and Aβ42 decreased. This was followed by an increased Aβ plasma/CSF ratio in the LCD1 group at day 51. In the immunized adult control dogs, plasma Aβ40 was also increased but remained constant in CSF. Therefore, an Aβ40 plasma/CSF increased ratio would represent a good marker of plasma clearance.

These modifications in plasma Aβ40 were accompanied by a 10-fold increased serum anti-Aβ40 IgG, with a peak at day 71 in CDS animals and at day 51 in adult control dogs. Only in this last group, anti-Aβ40 IgG was significantly increased in CSF at day 51, with an abundance of 0.01% of serum level, whereas in CDS animals, its increase was not significant. Compared to adult control dogs, the reduced immunogenic response in aged dogs, similar to humans [50], explain the delay and lower level of their serum IgG peak, and the discrete IgG presence in CSF. Anyway, the interrelationship of the processes taking place in CSF and blood seems to be similar in all groups of animals, as shown by the related temporal changes of amyloid parameters and IgG levels in both compartments to facilitate Aβ clearance. This is of special importance in view of the concern regarding the efficacy of immunotherapy in elderly patients with also a weak immune system [51].

The plasma anti-Aβ40 IgG and Aβ40 ratio of 1/25 was only found in immunized CDS dogs, suggesting the direct formation of antigen-antibody complexes to stimulate the sink effect from CNS to the periphery [52]. A variety of studies performed with different antibodies that recognize N and C terminal Aβ fragments argue for the same mechanism [53,54]. Because differences in antibody recognition of the Aβ peptide define its ability to cross the BBB, our v3 vaccine includes a variety of peptides that induce an antibody mixture to interact with the N or C Aβ terminal. So, the N-Aβ antibody recognition will mostly take place at the periphery, forming ag-ab complexes for the sink effect. Meanwhile, both N- and C-Aβ antibody recognition will favor the
central recognition of Aβ monomers, oligomers and mainly diffuse plaques, due to the maturation affinity process of B cells during the immunization period [55,56]. BBB damage is observed in many neuropathologies like AD and its permeability increases with aging. However, the lack of BBB alteration in CDS dogs may be related to the absence of mature plaques, or to LRP1 and RAGE modifications in their function of Aβ clearance [57–59]. In any case, with immunization, the BBB active Aβ removal would increase and facilitate cognitive improvement.

The v3 vaccine antigen builds a boost of antibodies, together with the possibility of a B-cell response by the C-Aβ epitope [60]. Irregular familiar feeding habits are considered the main cause of the dog higher phosphatase alkaline levels because of its role in the digestion and absorption of fats in the intestinal tract [61] and cannot be related to the immunization. The risk of an inflammatory response was also fully discarded by the clinical follow-up of all animals and the normality of the frequent plasma and CSF analyses.

CONCLUSIONS

In conclusion, we have designed and validated in old dogs with graded cognitive dysfunction and a weak immune system the efficacy and security of a new vaccine considered a good candidate for human AD prevention and treatment. This study also confirms familiar dogs as a good natural model of AD, with the aim of developing innovative and reliable therapies. In these animals, the behavioral, CSF and plasma studies are rapid and easy to perform, and allow to decipher the intrinsic mechanisms involved in Aβ physiopathology. This will shed light on our understanding of human amyloidosis.
ABBREVIATIONS

ag-ab = antigen-antibody
AC = Adult Control
AP = Alkaline Phosphatase
CDS = Cognitive dysfunction syndrome
CSF = Cerebrospinal fluid
EDTA = Ethylenediaminetetraacetic acid
i.v = intravenously
Qalb = Albumin quotient
LCD = light cognitive deficit
OC = Old Control
s.c = subcutaneously
SCD = severe cognitive deficit
DISCLOSURE STATEMENT

M.J.R., M.P and N.M. hold an EU patent (No. WO2010/012749) exploited by Medivet Pharma, s.l. and hold shares of the company. The other authors report no disclosure.

ACKNOWLEDGEMENTS

The study was supported by the Centre for Industrial Technological Development (NEOTEC Initiative) Ministerio de Ciencia e Innovación, Spain. Research by NM and MJR work was supported by the grants SAF2008-01902 and IPT-010000-2010-35 from the Ministerio de Ciencia e Innovación, and by the 2009SGR1380 grant from the Generalitat de Catalunya, Spain. We thank the Ars Veterinaria Hospital, Barcelona, Spain, for their help in the dog clinical assessment.

REFERENCES


FIGURE LEGENDS

**Fig. 1** Schematic timeline of canine immunization study. Horizontal arrow indicates the immunization period during which 6 doses of v3 vaccine or were subcutaneously injected. Vertical arrows indicate the cognitive evaluation days and the CSF and serum extraction days. The same immunization period and blood extraction protocol was used in the study performed in rabbits to select the best vaccine composition.

**Fig. 2** Plasma Aβ₄₀ and CSF Aβ₄₀ and Aβ₄₂ levels in control (AC and OC) dogs and CDS dogs (LCD and SCD). In LCD dogs, plasma Aβ₄₀ levels (A) are different (p=0.029) and also CSF Aβ₄₂ levels (C) (p=0.018). Two subgroups are observed in this LCD group: LCD1, the dogs with higher plasma Aβ₄₀ levels (p=0.009) and LCD2, the dogs with higher CSF Aβ₄₂ levels (p=0.018). CSF Aβ₄₀ level is similar in control and CDS dogs (B).

**Fig. 3** Anti-Aβ₄₀ levels in v1, v2 and v3 immunized rabbits during the immunization period (A) and cognitive changes of v3 immunized CDS dogs (B). Anti-Aβ₄₀ IgG levels in immunized rabbits reach a peak at day 51. Rabbits immunized with the v3 vaccine, present higher antibody levels. The dog cognitive changes during the immunization study was evaluated by the veterinarian with a validated Cognitive test performed at days 0, 31, 51, and 131. At day 31, significant changes were already detected in all immunized CDS dogs (p=*0.027) (B).

**Fig. 4** Plasma Aβ₄₀, CSF Aβ₄₀ and Aβ₄₂, and antibody levels during the whole study. A tendency to increase of plasma Aβ₄₀ in AC and CDS dogs (A), is observed but only a marked decrease of CSF Aβ₄₀ and Aβ₄₂ levels in CDS dogs after the immunization period (*p=0.049 for A β₄₂) (B,C). The ratio between those levels is only increased in the LCD dogs when we compare the groups at days 0 and 51, (H). Anti-Aβ₄₀ IgG is detected in serum and CSF at day 30 after the first boost (levels over 0.24nm) (E-F). In AC dogs the highest level is observed at day 51 (*p=0.0042 vs pre-immune levels;
*p=0.0079 vs placebo levels) (E) and in CDS dogs at day 71 (*p=0.0078 vs pre-immune levels; (*p=0.0022 vs placebo levels). CSF Anti-Aβ40 IgG is detected in all dogs with significant differences at day 31 in AC dogs (#p=0.0042 vs pre-immune values; *p=0.0078 vs placebo values) (F). Serum and CSF antibodies are positively correlated in AC and CDS dogs (G). However, plasma Aβ40 and plasma anti-Aβ40 IgG levels are only correlated in CDS dogs, (I).

**Fig. 5** Detection of Aβ plaques in AD human brain with purified antibodies from immunized rabbits and dogs. Micrographs at 5x and 40x. Antibodies from v1 (Aβ40) or v3 (Aβ40+ Aβx-40) immunized rabbits detect diffuse and core plaques (B, D). Antibodies from v2 (Aβx-40) immunized rabbits only detect diffuse plaques (C). Antibodies from v3 immunized dogs only detect diffuse plaques. Plaque detection with IgG from AC (F) or CDS dogs (G) is similar. Positive control with a commercial antibody (A, E).
Figures

Fig. 1

![Diagram showing Immunization period, Days, Cognitive test, Blood, CSF](image-url)
A. Anti-Aβ40 IgG in rabbit

![Graph depicting antibody response over time in rabbit serum extraction days.]

B. Cognitive test score

![Bar chart showing cognitive test scores over time for placebo and immunized groups.]

* Significant difference
Fig. 5

Dog anti-Aβ40

Rabbit anti-Aβ40
Table 1 Diversity of the dogs

<table>
<thead>
<tr>
<th>ID</th>
<th>Breed</th>
<th>Sex</th>
<th>Age</th>
<th>Screening group</th>
<th>Immunization group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>2</td>
<td>AC</td>
<td>Immunized AC</td>
</tr>
<tr>
<td>2</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>2</td>
<td>AC</td>
<td>Immunized AC</td>
</tr>
<tr>
<td>3</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>3</td>
<td>AC</td>
<td>Immunized AC</td>
</tr>
<tr>
<td>4</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>4</td>
<td>AC</td>
<td>Immunized AC</td>
</tr>
<tr>
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<td>Male</td>
<td>4</td>
<td>AC</td>
<td>Immunized AC</td>
</tr>
<tr>
<td>6</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>6</td>
<td>AC</td>
<td>Immunized AC</td>
</tr>
<tr>
<td>7</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>4</td>
<td>AC</td>
<td>Placebo</td>
</tr>
<tr>
<td>8</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>2</td>
<td>AC</td>
<td>Placebo</td>
</tr>
<tr>
<td>9</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>2</td>
<td>AC</td>
<td>Placebo</td>
</tr>
<tr>
<td>10</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>3</td>
<td>AC</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>6</td>
<td>AC</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Housed beagle dog</td>
<td>Male</td>
<td>8</td>
<td>OC</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Belgian Shepherd dog</td>
<td>Female</td>
<td>6</td>
<td>AC</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Rottweiler</td>
<td>Female</td>
<td>7</td>
<td>AC</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Mixed breed</td>
<td>Female</td>
<td>6</td>
<td>AC</td>
<td></td>
</tr>
<tr>
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<td>OC</td>
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<td>OC</td>
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</tr>
<tr>
<td>18</td>
<td>Mixed breed</td>
<td>Male</td>
<td>12</td>
<td>OC</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Catalan shepherd breed</td>
<td>Male</td>
<td>12</td>
<td>OC</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Beagle</td>
<td>Male</td>
<td>13</td>
<td>OC</td>
<td></td>
</tr>
</tbody>
</table>
12 housed beagle and 24 familiar dogs were used in the study. In this last group, 14 animals identified as CDS by their neurological records and cognitive test were subdivided into two groups according to their total score: 10 dogs identified with Light Cognitive Dementia (LCD) and 4 with Severe Cognitive Dementia (SCD), (See details in Table1). For their characterization, they were matched with Adult Control (AC, n= 14) and Old Control dogs (OC, n= 8). For the Immunization study 12 CDS dogs and 9 housed beagle dogs were included, and 9 CDS (Immunized CDS) and 6 housed beagle dogs (Immunized AC) received the v3 vaccine. 3 CDS dogs and 3 AC received Saline solution + Alum (Placebo group).
Table 2. Cognitive Test

<table>
<thead>
<tr>
<th>Items</th>
<th>Score</th>
<th>Items</th>
<th>Score</th>
<th>Items</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Walking</td>
<td></td>
<td>(4) Life rhythm</td>
<td></td>
<td>(7) Learned specific behavior</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>Normal</td>
<td>1</td>
<td>Normal (unchanged)</td>
<td>1</td>
</tr>
<tr>
<td>Trudging</td>
<td>3</td>
<td>Trudging</td>
<td>2</td>
<td>Random responses</td>
<td>3</td>
</tr>
<tr>
<td>Abnormal, one direction, circling</td>
<td>5</td>
<td>Hypersomnia</td>
<td>3</td>
<td>Decreased response</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Restless at bedtime</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) Posture/ emotional of expression</td>
<td></td>
<td>(5) Play behavior</td>
<td></td>
<td>(8) Adaptive capabilities</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>Normal</td>
<td>1</td>
<td>Normal</td>
<td>1</td>
</tr>
<tr>
<td>Decrease of body language</td>
<td>3</td>
<td>Increase</td>
<td>3</td>
<td>Retreats from novel situation</td>
<td>2</td>
</tr>
<tr>
<td>Abnormal, loss of body language</td>
<td>5</td>
<td>Decrease</td>
<td>5</td>
<td>Unable to accept changes in routine</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Looks indifferent to changes</td>
<td>5</td>
</tr>
<tr>
<td>(3) Elimination behavior</td>
<td></td>
<td>(6) Exploratory behavior</td>
<td></td>
<td>(9) Interaction with other animals or with owners</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>Normal</td>
<td>1</td>
<td>Normal</td>
<td>1</td>
</tr>
<tr>
<td>Defecate and urinate at home:</td>
<td>3</td>
<td>Decrease (disorientation)</td>
<td>3</td>
<td>Decreased (loss of relationship)</td>
<td>3</td>
</tr>
<tr>
<td>occasionally in a small scattered amount</td>
<td>4</td>
<td>Decrease (disorientation)</td>
<td>3</td>
<td>Decreased (loss of relationship)</td>
<td>3</td>
</tr>
<tr>
<td>Loss control of sphincters:</td>
<td>4</td>
<td>Decrease (disorientation)</td>
<td>3</td>
<td>Decreased (loss of relationship)</td>
<td>3</td>
</tr>
<tr>
<td>defecate and urinate outside of sleeping area</td>
<td>5</td>
<td>Decrease (disorientation)</td>
<td>3</td>
<td>Decreased (loss of relationship)</td>
<td>3</td>
</tr>
<tr>
<td>Loss control of sphincters:</td>
<td>5</td>
<td>Decrease (disorientation)</td>
<td>3</td>
<td>Decreased (loss of relationship)</td>
<td>3</td>
</tr>
<tr>
<td>defecate and urinate inside of sleeping area</td>
<td>5</td>
<td>Decrease (disorientation)</td>
<td>3</td>
<td>Decreased (loss of relationship)</td>
<td>3</td>
</tr>
</tbody>
</table>

Total score in the 9 items test: <12 = normal; 12-26 = light cognitive deficits SCD; >26 = severe cognitive
Table 3. Hematological and biochemistry parameters during immunization study

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Placebo</th>
<th>Immunized AC</th>
<th>Immunized CDS</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cells</td>
<td>6.49±0.68-10.88±1.58</td>
<td>6.08±0.99-11.10±0.416</td>
<td>6.99±1.15-0.86±1.26</td>
<td>5.05-16.76</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.30±0.15-2.20±0.66</td>
<td>1.05±0.16-2.33±0.74</td>
<td>1.07±0.21-2.30±0.54</td>
<td>1.05-5.10</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.42±0.81-1.18±0.19</td>
<td>0.45±0.11-0.98±0.15</td>
<td>0.43±0.05-1.04±0.15</td>
<td>0.16-1.12</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.38±0.50-13.00±8.52</td>
<td>4.21±0.52-8.06±0.13</td>
<td>4.18±1.59-6.67±1.38</td>
<td>2.95-11.64</td>
</tr>
<tr>
<td>Eosinophil’s</td>
<td>0.22±0.05-0.37±0.10</td>
<td>0.31±0.04-0.72±0.16</td>
<td>0.19±0.08-0.43±0.11</td>
<td>0.06-1.23</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.02±0.004-0.06±0.01</td>
<td>0.02±0.005-0.06±0.01</td>
<td>0.02±0.006-0.05±0.01</td>
<td>0.00-0.10</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>46.88±2.51-48.83±1.07</td>
<td>47.06±1.46-57.90±1.13</td>
<td>41.27±4.73-47.82±6.57</td>
<td>37.3-61.7</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>14.45±0.48-16.13±0.55</td>
<td>14.96±0.41-18.63±0.41</td>
<td>13.70±1.35-5.27±0.56</td>
<td>13.1-20.5</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>29.86±8.71-42±4.80</td>
<td>37.80±4.08-64.13±9.95</td>
<td>29.81±4.49-34.41±7.93</td>
<td>6.6-100.7</td>
</tr>
<tr>
<td>Platelets</td>
<td>178.33±31.07-381.25±84.86</td>
<td>221.67±5.17-18.33±25.20</td>
<td>285.50±40.77-343±41.51</td>
<td>148-484</td>
</tr>
<tr>
<td>Biochemical parameters</td>
<td>Placebo</td>
<td>Immunized AC</td>
<td>Immunized CDS</td>
<td>Range</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------</td>
<td>--------------</td>
<td>---------------</td>
<td>--------</td>
</tr>
<tr>
<td>Alkaline Phosphatase (AP)</td>
<td>105.33±6.32</td>
<td>89.33±18.50</td>
<td>403.71±136.59-760.71±256.45</td>
<td>23-212</td>
</tr>
<tr>
<td>Glutamate Pyruvate Transaminase (GPT)</td>
<td>41.33±9.49</td>
<td>39.67±9.82</td>
<td>99.66±22.44</td>
<td>0-100</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.79±0.11-1.10±0.07</td>
<td>0.79±0.08-1.04±0.02</td>
<td>0.87±0.11-1.13±0.15</td>
<td>0.5-1.8</td>
</tr>
<tr>
<td>Urea</td>
<td>33.25±3.62</td>
<td>24.43±3.76</td>
<td>40.52±8.98-106.21±36.67</td>
<td>18-60</td>
</tr>
<tr>
<td>Total protein</td>
<td>6.20±0.115-6.66±0.287</td>
<td>5.90±0.06-6.80±0.15</td>
<td>6.55±0.13-7.45±0.21</td>
<td>5.2-8.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>100.50±12.41-115.66±6.35</td>
<td>98.00±10.01-128±6.50</td>
<td>89.80±4.54-107.28±5.35</td>
<td>74-143</td>
</tr>
</tbody>
</table>

Biochemical and hematological parameters measured during the immunization study with their minimum and maximum values (±s.d.). Values in bold of alkaline phosphatase present significant differences in familiar dogs.