Project Acronym:	**LIPIDIDIET**
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1 Executive summary

When LIPIDIDIET was designed in 2006, first indications surfaced that treatment of Alzheimer's disease (AD) would be most effective if applied early. Moreover, the first publication appeared in that year showing how AD could be identified before the onset of dementia symptoms (prodromal AD). These advances enabled focus on the prodromal phase of the disease. Considering the very mild symptoms present at this stage, any intervention to be developed should be as safe to use as possible. From our previous studies (e.g. LIPIDIET) the neuroprotective docosahexaenoic acid (DHA), in combination with other nutritional molecules, presented the most suitable option.

Main Objectives: to develop effective nutrition for AD prevention; to study the effectiveness within a prospective clinical trial; to decipher the molecular pathways; to develop more effective second generation diets; to identify diet related epidemiological risk factors for dementia; and to make the spectrum of interventional steps available for the benefit of the aging EU population.

Main results: LIPIDIDIET developed multiple analytical methods which can now be used to gain more detailed results than before and to achieve readouts for parameters which were previously not accessible. The mechanism by which DHA is neuroprotective has been comprehensively studied. It was found to enhance a large variety of molecular mechanism which acts in combination to result in significant neuroprotection. This includes pleiotropic action within a single pathway or combination of several neuroprotective pathways. For example, the initial event in AD aetiology is overproduction of Amyloid beta (Aß) peptides, which reduces synaptic function in neurons. DHA decreases Aß peptide production through alteration of 13 different cellular pathways. Unanimously these changes reduce Aß production. Furthermore, DHA increases synapse and blood brain barrier function and reduces inflammatory signalling and reactive oxygen species. DHA 'corrects'

several of the dysfunctional pathways affected by apoE4, the major genetic risk factor for AD. Lipid pathways affected in AD, were identified and found to be addressable by dietary intervention. Furthermore, lipids which increase AD risk, were identified and potential dietary alternatives to those discovered. Over the course of the project it has become evident that, while DHA has effects on AD relevant mechanisms, combined nutrient administration including B-vitamins, uridine, choline, anti-oxidants, and phospholipids does significantly enhance the effects observed. Some of the benefits (e.g. some of the vascular and brain structural benefits) were absent or little present with DHA alone and clearly depended on the co-administration of the other nutrients. Several alternative nutritional concepts yielded similar or less positive results. Overall, within the studied disease scenario, the best diet was found to be Fortasyn Connect. Importantly, for some of the alternative diets we observed unexpected adverse effects, highlighting the need for preclinical studies (as done within LIPIDIDIET) before evaluation in clinical trials.
The LIPIDIDIET clinical trial is one of the first randomized controlled trials (RCTs) with 2 years follow-up of patients with prodromal AD. Response by the participating volunteers has been very positive: the study drink containing the most effective multi-nutrient combination identified is well received and very few participants have thus far opted to leave the trial before finishing the first two year milestone. Indeed, the first patients are already in their sixth year of continuous treatment. Detailed outcome results will be available through scientific and general public media.
To make recommendations for brain health related to diet we developed a 'healthy brain diet' index and a mid-life risk score. Our data show that over-nutrition is not healthy for the brain, especially in mid-life. Detailed information on specific dietary patterns is provided as a guide to a healthy diet published on the LIPIDIDIET website.
LIPIDIDIET is the first study which comprehensively studied the impact of multi-nutrient intervention in AD prevention, spanning the full range from molecular design, pathway analysis, in vivo evaluation, epidemiological evidence, full scale long-term interventional clinical trial, to having the actual product and dietary advice, all based on a sound, solid and externally evaluated scientific basis, available for the general consumer. Approaches taken by LIPIDIDIET are already being used as a model for related and unrelated areas of research. LIPIDIDIET research continues within the JPI/JPND project MindAD, the PDP and others to generate personalized and multidimensional dementia prevention and to implement dementia prevention within the European health services.
At this time, LIPIDIDIET has published over a hundred of peer-reviewed scientific journal publications as well as being featured in many public press, TV and radio interviews.

2 Description of the project context and objectives

Major objective: It is our aim to develop a lipid based diet that is able to delay or prevent the onset of AD and related diseases and has a stabilizing effect on cognitive performance in aging.

Whom we aim to treat and why: Over the recent years it has become increasingly evident, that treatment of AD dementia has clear limitations in what can realistically be achieved in the foreseeable future. The general view now is that, due to the massive neurodegeneration already present once AD has progressed to the dementia stage, a cure from the disease would be very difficult to achieve. However, since AD develops slowly over a time-course of several decades, and since neuronal damage is rather low throughout most of this time, an early intervention is now generally assumed to offer the best chances to slow down, stop or reverse the AD disease process. In LIPIDIDIET a screening procedure was used to identify those in the population who were highly likely to develop AD dementia, but the disease did not (yet) progress to the dementia phase of the disease. This is based on a combination of biomarkers and cognitive assessment. Since LIPIDIDIET started, this diagnostic approach has been verified to allow the identification of the prodromal AD stage by multiple other studies conducted by external investigators as well as LIPIDIDIET Consortium members.

How: Multiple lines of evidence suggest that there is a large overlap between risk factors for AD, VaD (Vascular Dementia) and cognitive decline in the elderly. Importantly, there is equally strong evidence that prevention and treatment of these diseases can be efficiently addressed - particularly in their initial and preclinical stages - by closely related or identical bio-molecules. Predominantly, these molecules appear to belong to the class of lipids that are part of the human diet. However, very often they are consumed in far lower than recommended amounts. Bearing in mind that these diseases have a long pre-clinical phase, in which the disease remains undetected,

specifically designed nutrition may be required for effective prevention or for those who have already progressed into the first clinical stage of the disease. Moreover, within the elderly population pathological changes occur frequently not due to one disease (AD, VaD and pathological aging related changes), but by two or all of these that occur in combination. Thus targeting only one would be insufficient. Taking these aspects into consideration, dietary supplementation, composed to maximize benefit for all three of these in the elderly common diseases, appears to be the most suitable approach to provide a general health perspective improvement for this age group in the EU population.

The LIPIDIDIET project is based on our scientific successes and developments within the previous LIPIDIET project (QLRT-2001-00172). In recent years, we and others have shown that lipid-based nutrition significantly influences dementia-related pathology. Screening large numbers of lipids and fatty acids we found DHA to be the most effective single substance to reduce amyloid production/burden *in vitro* as well as *in vivo*. In 2006 we completed a small fish-oil (DHA/EPA) treatment trial of patients with very mild Alzheimer's disease and observed that disease progression in the treated patients was reduced. However, the treatment was not effective in more advanced stages of AD. These results showed that synergistic and additive effects can be obtained by putting together the appropriate combination of certain nutrients which clearly outperform that of individual nutrients, especially in the context of the neurodegenerative processes studied here.

Qualification – The Consortium – For this research project, experts from all over Europe have come together to form a Consortium covering the multiple research fields and expertises necessary to conduct this demanding research project. Several participants are members of the previous LIPIDIET project. All partners have long standing first-rate experience and renowned international reputation in their fields.

Main Objectives: We will use our longstanding and broad research experience to further investigate the role and function of lipids in aging, Alzheimer's disease and vascular dementia

- to **develop nutrition** which is more effective than the dietary supplements currently available for AD prevention,
- to study the disease prevention potential of lipid-based nutrition within a **prospective clinical trial**. For this, we will build on the results of the LIPIDIET project, using the latest diet developed in that framework, and go beyond, now testing that diet in a clinical trial,
- to decipher the **molecular pathways** in the processes and to develop more effective second generation diets,
- to evaluate effectiveness and identify environmental and other **epidemiological risk factors for dementia**,
- **to make** the spectrum of interventional steps **available** for the benefit of the aging EU population, spanning from **dietary advise** to actual **dietary products** which will address **private, clinical and nursing homes** needs in nutritional intervention for **dementia** and the preservation of **cognitive performance in normal aging**.

Relevance – Objectives – Our objective is to improve the health and quality of life of the ageing population by addressing nutritional issues that affect the ageing process, based on solid scientific evidence and the important progress in research which has been made especially during the recent years. Our primary clinical target group of patients, suffering from prodromal dementia/MCI-AD, is especially suitable because it represents the first symptomatic manifestation of Alzheimer's disease. Issues such as understanding the beneficial and harmful dietary effects of lipids or lipid-related diets on the development of age-related disorders, addressing the specific needs and habits of the aging and older population groups, which are at a very high risk for dementia and vascular pathology, as well as shedding light on the mechanisms underlying the age-related loss in cognitive performance are of highest importance, globally. Diet is a modifiable risk factor in the development of diseases like Alzheimer's disease and vascular dementia. Furthermore, it offers decisive means of effective, acceptable and affordable interventions which can potentially influence the course of disease.

Technical objectives

I) **To provide technical support for the LIPIDIDIET project.** The LIPIDIDIET project has strong cross-faculty expertise covering a variety of scientific disciplines. This project work package (WP) combines those areas of expertise which are of a technical nature and have high demands on available technology, know-how and materials which cannot effectively be transferred between partners.

II) **To develop and produce a) experimental rodent diets for preclinical research and b) test compositions for a clinical trial and for human consumption.** The experimental rodent diets are based on a standardized control diet to allow comparison, modified by the results of the *in vitro* and animal studies, as well as on the epidemiological data. These data provide lists of most and least preferred dietary components for the induction of health benefits for dementia patients and the elderly. There were two successive cycles of diets. The first cycle incorporates the results of previous work by the Consortium, whereas the second cycle was based on the results obtained within the LIPIDIDIET project. The standardized control rodent diet we produce meets the dietary requirements of rats and mice concerning both macronutrients and micronutrients. Based on this standardized control diet, several adjusted diets were formulated.

III) **To identify the mode of action (MOA) or combinations of MOA which are responsible for the beneficial effect observed for DHA-based nutrition on neuroprotection and cognitive performance stabilization in animals and humans.** In order to develop most effective health-targeted nutrition one needs to identify and understand the parameters which govern the biological principles by which nutrition exerts its effects, in our case neuroprotective properties of lipid-based nutritional formulations. A major challenge of this WP is that many different MOAs have been described for DHA. All of these are based on rational approaches which were proposed to sufficiently explain the observed outcomes, but it is was not possible to identify which MOA, or combinations of MOA, were actually responsible for the neuroprotective property. No comprehensive study systematically comparing the proposed MOA of DHA in neuroprotection had been conducted thus far. This would, however, be of utmost importance, because identification of the beneficial mechanism would specifically address this mechanism to further increase effectiveness of the treatment/nutrition.

IV) **To enhance the effectiveness of nutritional formulations on neuroprotection.** A very important aspect of the development of DHA-based nutrition is to increase the effectiveness. It has been assumed that currently used fish-oil based applications are sub-optimal and that more complex formulations would increase effectiveness. Some of these principles are already implemented in the second generation nutrition resulting from LIPIDIET. Moreover, with some other lipids there was no obvious influence on Aß generation, but when applied in combination they reduced the Aß lowering capacity of Aß lowering lipids *in vitro*. The aims here were to A) identify the lipid-related factors which increase the effectiveness of DHA-based nutrition on neuroprotection, B) evaluate the dietary fat blends and individual lipids of FA for their interaction with potentially effect reducing lipids, C) use the information on DHA MOA to develop more effective nutritional compositions D) develop prototype diets with increased effectiveness for *in vivo* testing based on the aforementioned studies.

***in vivo* evaluation**

V) **Animal model Amyloid –** To directly test the efficacy of new lipid-based test diets to slow down amyloid accumulation and development of memory impairment. Amyloid, in particular soluble toxic oligomers, are believed to cause Alzheimer's disease. This WP gave the *in vivo* result of the effect of the diets on amyloid parameters. It does not contain as much mechanistic investigations, since those are done in other WPs. Analysis parameters are those which have proven to be the best indicators for neuroprotection. These are (soluble) Aß levels, Aß species, ratios and truncations and of course cognitive performance/ behaviour.

VI) **Animal model Apolipoprotein E (apoE) – To directly test the ability of the diets to prevent, delay or reverse the neuropathological and cognitive effects of apoE4.** ApoE4 is the major genetic risk factor for Alzheimer's disease and is also an important risk factor for some other chronic neurodegenerative diseases, for acute brain injury and stroke. Analysis parameters were those which have proven to be the best apoE-related indicators for neuroprotection. These are neuronal plasticity impairments of apoE4 and the counteraction by diet of apoE4-induced aggravation of amyloid pathology.

VII) **To evaluate the cerebrovascular effects of lipid-based nutrition.** There are many indications that cardiovascular disorders play a much larger role in the etiology of neurodegeneration than initially thought. It is important to increase knowledge of the reason why some people with hypercholesterolemia and other cardiovascular disorders develop neurovascular degeneration and cognitive impairment, while others don't. It was hypothesized that vascular disorders result in cognitive impairment and dementia via impairment of blood vessels and circulation in the brain and decreased energy metabolism, causing white matter lesions and the production of amyloid leading to further exacerbation of this neurodegenerative processes in aging.

Human objectives

In the end, one can only find out whether nutrition has actual health benefits for humans if it is evaluated in a clinical trial or studied in epidemiology. Both methods have their advantages and potential drawbacks. Epidemiological studies allow identification of various risk factors with relative ease, but they can report only on correlations, which are easy to misinterpret. Actual effects on health by a given substance or combination of substances can only be definitely proven by performing the appropriately designed prospective clinical trial. Without the latter no final conclusions can ever be drawn for the impact of an intervention on people.

VIII) **Clinical trial – to investigate the effect of supplementation with omega-3 fatty acid enriched with other nutrients in prodromal Alzheimer's disease, double blind placebo/control controlled study on cognition, biomarkers in plasma, CSF and atrophy rate on MRI.** Through our previous work, a randomized, double blind, placebo-controlled 1 year study in patients with mild to moderate AD, was conducted to evaluate the cognitive effects, tolerability and safety of dietary supplementation with n-3 fatty acids on stable doses of AchE inhibitors. In this trial, n-3 fatty acid supplementation correlated with positive effects, which were observed in the subgroup of patients with very mild AD implying that patients with prodromal AD should be included in future studies. Building on this, the current project involves the prodromal AD population, commonly elderly people who have not yet developed symptoms of AD, but will over time progress to this disease. Recent advances in early AD diagnosis have made it possible to identify this group in a more accurate way than ever before. Moreover, this population is the best suited to investigate the prevention potential of diets and enables several aspects of the effect of nutrition on *healthy* aging to be addressed at the same time. Patients were given the second generation nutrition (Fortasyn Connect) and studied for a minimum of 2 years using well-established cognitive-, pathological-, and biological -markers.

IX) **Epidemiology** – In Sweden and Finland, comprehensive, longitudinal studies of aging have been conducted since 1968. The LIPIDIDIET Consortium had access to these study populations and databases. These study populations are particularly suitable for detecting lifestyle- and dietary habit-related risk factors which need considerable time to result in observable effects. These studies will now be extended to investigate blood cholesterol/lipid levels and AD and cerebrovascular disease in mid- and late-life, adiposity factors and AD and cerebrovascular disease in mid- and late-life, dietary fat intake and habits in mid-life and AD and cognitive impairment, fruit and vegetable intake in mid-life and AD and cognitive impairment, the potential modifying role of apoE in relationship to AD and cerebrovascular diseases and the relationship to blood cholesterol levels and adiposity indices, and dietary intake, and AD and cerebrovascular disease. From these data, a 'healthy brain diet' index was developed, which takes into account the relationship of diet to AD and cognitive impairment. Furthermore, – dietary recommendations and a mid-life risk score for AD and cognitive impairment based on diet and blood levels of 'fat' factors are proposed.

3 Description of main S&T results/foregrounds

The project is organized in thematic work packages (WPs) and within each WP up to 11 different research groups work together to achieve the main tasks of this research project.

WP1 - Technical support

The objective of this WP is **to provide technical support for the LIPIDIDIET project.**

The LIPIDIDIET project has strong cross-faculty aspects covering various scientific disciplines and areas of expertise. This WP combines those areas of expertise that are of a technical matter and have high demands on available technology, know-how and materials which cannot effectively be transferred between partners.

Six research groups, all with different expertise provided essential technical support to 16 partners of LIPIDIDIET. Within this WP three new methodologies were developed (phospholipids, sterols, Aß aggregation), e.g. to identify and quantify lipids more effectively, were developed. Together the six WP partners performed thousands of highly sophisticated assays for the Consortium.

Some of these assays are rather unique, like some in-situ membrane enzyme assays, and some have been developed specifically for the LIPIDIDIET project, while others require very specific instrumentation or highly complicated methodology necessitating years of experience.

Considering the vast amount of data produced by WP1, this WP continued to not only produce far more output than expected, but also greatly contributed to increasing the scientific quality and depth of the entire LIPIDIDIET project. Further assays were done and a new cellular model to study Alzheimer's disease had been developed.

There now follow some examples of novel methods that were introduced and used for both qualitative and quantitative analysis of phospholipids from different biological samples:

1.) Development of a new and rapid LC-MS method based on MS^E scan mode
2.) Analysis of phospholipids with combination of HPLC-ESI-QTOF and UPLC-ESI-QTOF
3.) Application of LC-ESI-MS(MS) for phospholipid analysis of brain extracts
4.) Multidimensional LC-technique for MS analysis of phospholipids
5.) Development of a novel method for lipid analysis with hydrophobic interaction chromatography (HILIC).

Phospholipid analysis of brain samples proved to be a very useful tool for controlling the effect of different diets in animal experiments. New methods for lipid analysis of biological samples were developed and introduced: New generation HPLC columns (Kinetex) provided very good resolution of phospholipids in complex biological samples.

For separation of phospholipids in brain samples a new chromatographic method was developed. With this method many different phosphatidyl-serine, different phosphatidyl-ethanolamine and phosphatidyl-choline species were identified.

For controlling the effect of different diets, a large number of brain samples (from tg and wt mice after different diets) and of different brain regions were analysed for phospholipids.

For accelerating the analytical control of the *in vivo* experiments, novel methods of lipid analysis of biological samples were developed and introduced:

- Phospholipid (PL) analysis using RP-HPLC on C-IP column combined with electrospray ionization mass spectrometry. The method was used in determination of PL composition changes of mouse brain (hippocampus and cortex) samples after fish oil, Fortasyn and cholesterol/phytosterol enriched diets.
- We developed a new method for offline 2-dimensional LC-MS separation of different phospholipids from different samples. First dimension is hydrophilic interaction chromatography (HILIC) of PL classes; the second dimension is the RP-HPLC separation of PL species of the PL classes.

Hydrophobic interaction chromatography (HILIC) and zwitter ionic stationary phases (ZIC-HILIC) as new methods were used for qualitative and quantitative analysis of ceramides (CE) and sphingomyelines (SM), the key compounds for diverse biological processes.

We also established highly sensitive and specific isotope dilution methodologies for the measurement of sterols, stanols and oxysterols in serum, tissues (brain homogenates, hippocampus, cortex, liver) and cerebrospinal fluid. Sterols and oxysterols are measured by gas chromatography-mass spectrometry- selected ion monitoring using deuterium labelled sterols and

oxycholesterols and oxyphytosterols. For this purpose authentic and stable isotope (deuterium) labelled compounds were synthesized. The labelled compounds are used either as internal standards or tracer compounds in kinetic experiments.

Other methodology developed, advanced or applied for LIPIDIDIET WP1 includes e.g. assays for biomarkers, in situ secretase activity assays, other enzyme activities as well as standard assays. For more information on these we refer to the LIPIDIDIET publication list.

WP2 - Diet generation

The major objective of the LIPIDIDIET project was to develop a lipid based diet that is able to delay or reduce risk of onset of Alzheimer's disease and related diseases and has a stabilizing effect on cognitive performance in aging. Building on important findings from the preceding LIPIDIET project, showing that specific lipids may have beneficial effects on Alzheimer pathology and cognitive function, routes towards more effective combinations of nutrients were to be investigated. Within the current project, for WP02 the main objectives were **to develop and produce a) experimental rodent diets for preclinical research and b)** liquid nutritional compositions for the randomized controlled trial in persons at high risk of Alzheimer's disease (WP08).
In close collaboration with analytical and mechanistic WPs, which provided data on additive and synergistic effects of different nutrients, various cycles of dietary compositions were developed for further scientific evaluation.

The experimental rodent diets are based on a standardized control diet to allow comparability, modified by the results of the *in vitro* and animal studies, as well as on epidemiological data. These data provide lists of the most and least preferred dietary components for the induction of health benefits for dementia patients and the healthy elderly. There were two successive cycles of diets. The first cycle incorporated the results of our previous (FP5 LIPIDIET) and other works, whereas the second cycle is based on the results obtained within this project. The standardized control rodent diet we produce meets the dietary requirements of rats and mice concerning both macronutrients and micronutrients. Based on this standardized control diet, several adjusted diets were formulated. Specific isocaloric formulations were developed. The products were then distributed to the clinical and preclinical research groups for efficacy evaluation in the respective animal models and for use in the clinical trial. Several experimental diets as well as the clinical active and control diets were produced.

As a base for the development of experimental diets within the LIPIDIDIET project, a standardized control diet was formulated that provided all necessary nutrients for normal development and growth. For comparability, all experimental diets that were developed over the course of the project were based on the control diet and were isocaloric, i.e. provided equal amounts of energy to the subject. Based on the important role of dietary lipids like the omega-3 long chain poly-unsaturated fatty acid DHA, together with previous experimental and epidemiological evidence, more complex nutritional compositions were formulated. After production, diets were distributed to the respective experts in the Consortium for scientific evaluation of their efficacy on parameters relevant to AD.

Over the course of the current project, different cycles of experimental diets were developed and tested. There were two main successive cycles of diets. The first cycle incorporated the results of our previous (FP5 LIPIDIET) and other works, whereas the second cycle is based on the results obtained within this project. Mechanistic experimental findings showed significantly increased efficacy of combined nutrient supplementation, while mode-of-action related experimental data indicated efficacy on a whole range of processes relevant for the development of AD. During the project it became clear that, while DHA has positive effects on several mechanisms relevant for AD, the combined administration of DHA with other nutrients like choline, UMP, vitamins B6, B12, folate, phospholipids, vitamins C, E, and selenium does significantly enhance the effects observed. Synergies and additive effects were confirmed in several studies with different cycles of diets, up to a certain maximum effect; yet further enrichment of nutritional concepts yielded similar or somewhat less positive results. Together these data suggest that we have indeed developed a more effective lipid-based nutritional composition with a close to optimal neuroprotective profile. Recent scientific publications on the nutritional status of early Alzheimer patients confirm that especially in the abovementioned nutrients relative insufficiencies are present, confirming an increased requirement for these specific ingredients of our nutritional composition.

Based on the complex DHA-containing formulations that were tested, a liquid clinical product was developed, together with a control product that did not contain the combination of active ingredients. The result is a medical food product consisting of a 125 mL once-a-day drink containing the specific nutrient combination Fortasyn Connect (including omega-3 fatty acids, UMP, choline, vitamins B6, B12, folate, phospholipids, vitamins C, E, and selenium). The control product for the control group was a drink that was similar in appearance, flavour and calories, but without Fortasyn Connect. Participants in the human trial were randomly assigned to either the medical food or the control group. Participants, doctors, nurses, and psychologists evaluating the participants did not receive information about which group the participant belonged to (double-blind design).

For product development a selection of approved raw materials was made in close collaboration with world leading suppliers, yielding stable key ingredients with good emulsion stability. Flavour optimisation for the products was followed by evaluation by expert taste panel in order to select the best appreciated product concepts for production.

WP3 - Mechanism of action (MOA) of DHA

The objective of this WP is to identify the MOA or combinations of MOAs which are responsible for the beneficial effect of DHA-based nutrition on neuroprotection and cognitive performance stabilization. This WP is the basic research WP for identifying and understanding the parameters which govern the effectiveness of lipid-based nutritional formulations to exert neuroprotective properties. It aims to closely collaborate with the WP4 focussing on enhancing the effectiveness of the nutritional formulations and with the animal WP's (WP5, WP6, WP7) from which results and information are fed back for the successive cycles of development. The major concern of this WP is that many MOA have been described for DHA. All of these MOA are based on rational approaches which may sufficiently explain the observed outcomes; however, it is not yet possible to identify which MOA- or which combination of MOAs - is/are actually responsible, but we already clearly see that some MOA are apparently more relevant towards neuroprotection than others and that indeed DHA is effective because of the combinatory action of some of these effects.

Lipidic & amyloidogenic pathways. A comprehensive study was performed to analyse the impact of DHA on the regulation of the **amyloidogenic processing of APP**. According to our previous results DHA would be expected to reduce Aß production. This was confirmed for neurons. Amyloidogenic secretase activity is reduced, but the DHA MOA does not involve reduced presenilin protein levels as cause for reduced Aß production. Relevance of reduced secretase activity is confirmed with ex vivo prepared membranes, which show significantly reduced brain membrane secretase activity. This suggests that DHA directly reduces the amyloidogenic potency of the brain. DHA decreases Aβ production in multiple ways. In presence of DHA amyloidogenic processing is decreased by a reduced β- and γ-secretase activity, whereas the protein and expression levels of BACE1 and PS1 are unchanged. Non-amyloidogenic processing is increased by an elevated ADAM17 protein level, caused by decreased protein degradation and increased expression level. Besides the direct effect on APP processing, DHA affects Aβ production by a crosslink to cholesterol homeostasis. DHA decreases cholesterol *de novo* synthesis by decreasing HMGCR activity, whereas HMGCR protein level and expression are unchanged. Besides the reduced cholesterol production DHA causes a shift of cholesterol from the raft to the non-raft fraction which is accompanied by a shift in γ-secretase activity and PS1 protein. On each of the depicted "switches" DHA has a small to moderately strong impact, but always sets the switch towards the non-amyloidogenic setting. Since DHA sets all of these switches in parallel, the summation of these small effects results in a strong downregulation of amyloidogenic in favour of non-amyloidogenic processing.

Non amyloidogenic processing. An increase in α-secretase activity is an attractive therapeutic target for AD treatment. APP and the APP-cleaving secretases are all transmembrane proteins, thus local membrane lipid composition is proposed to influence APP processing. Although several studies have focused on γ-secretase, the effect of the membrane lipid microenvironment on α-secretase is poorly understood. The effect of fatty acid (FA) acyl chain length (10:0, 12:0, 14:0, 16:0, 18:0, 20:0, 22:0, 24:0), membrane polar lipid headgroup (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine), saturation grade and the FA double-bond position on α-secretase activity was systematically investigated. We found that α-secretase activity is

significantly elevated in the presence of FAs with short chain length and in the presence of polyunsaturated FAs, whereas variations in the phospholipid headgroups, as well as the double-bond position, have little or no effect on α-secretase activity. Overall, this study showed that local lipid membrane composition can influence α-secretase activity and may have beneficial effects for AD.

AICD signalling pathway. Alzheimer´s disease is characterized by an accumulation of Aß, released by sequential proteolytic processing of the amyloid precursor protein (APP) by ß- and γ--secretase. Aß peptides can aggregate, leading to toxic Aß oligomers and amyloid plaque formation. Aß accumulation is not only dependent on *de novo* synthesis but also on Aß degradation. Neprilysin (NEP) is one of the major enzymes involved in Aß degradation. Here the molecular mechanism of NEP regulation, which is up to now controversially discussed to be affected by APP processing itself was investigated. NEP expression was found to be highly dependent on the APP intracellular domain (AICD), released by APP processing. Mouse embryonic fibroblasts devoid of APP processing, either by the lack of the catalytically active subunit of the γ--secretase complex (presenilin (PS) 1/2) or by the lack of APP and the APP-like protein 2 (APLP2), showed a decreased NEP expression, activity or protein level. Similar results were obtained by utilizing cells lacking a functional AICD domain (APPΔCT15) or expressing mutations in the genes encoding for PS1. AICD supplementation or retransfection with an AICD encoding plasmid could rescue the down-regulation of NEP further strengthening the link between AICD and transcriptional NEP regulation, in which Fe65 acts as an important adaptor protein. Especially the AICD generated by the amyloidogenic pathway seems to be more involved in the regulation of NEP expression. In line with this, analysis of NEP gene expression *in vivo* in six transgenic AD mouse models (APP and APLP2 single knock-outs, APP/APLP2 double knock-out, APP-swedish, APP-swedish/PS1Δexon9 and APPΔCT15) confirmed the results obtained in cell culture.
This demonstrated an AICD-dependent regulation of the Aβ-degrading enzyme NEP *in vitro* and *in vivo* and serves to elucidate the underlying mechanisms that might be beneficial to develop new therapeutic strategies for the treatment of AD.

Serin-palmitoyl transferases. Changes in sphingolipid metabolism have been associated to the development of AD. The key enzyme in sphingolipid *de novo* synthesis is serine-palmitoyl-CoA transferase (SPT). Here we identified a new physiological function of APP in sphingolipid synthesis. The APP intracellular domain (AICD) was found to decrease the expression of the SPT subunit SPTLC2, the catalytic subunit of the SPT heterodimer, resulting in decreased SPT activity. AICD function was dependent on Fe65 and SPTLC2 levels are increased in APP knock-in mice missing a functional AICD domain. SPTLC2 levels are also increased in familial and sporadic AD *post mortem* brains, suggesting that SPT is involved in AD pathology.
For this we conclude: The AICD down-regulates serin-palmitoyl transferase activity, reducing sphingolipid synthesis and AGPS activity, a key enzyme for plasmalogen synthesis, reducing anti-amyloidogeneic and anti-oxidative potency.
Phytosterols are the molecular equivalents to cholesterol in plants with very similar chemical structures to that of cholesterol. We investigated the effect of the most prevalent plant sterols – stigmasterol, β-sitosterol, brassicasterol, campesterol - and cholesterol on APP processing by analyzing APP cleavage products, enzyme activities involved in APP processing, their gene expression and protein levels. We found that the plant sterols are not as amyloidogenic as cholesterol (amyloidogenic potential was ranked: stigmasterol<brassicasterol/campesterol<β-sitosterol<cholesterol). Importantly, stigmasterol significantly reduced Aβ generation. The analysis of the molecular mechanisms showed that stigmasterol directly inhibited β-secretase activity, whereas γ-secretase activity is not affected directly but rather by indirect mechanisms: gene expression and cholesterol distribution to lipid rafts. Experiments in mice fed with stigmasterol enriched diets confirmed protective effects of stigmasterol *in vivo*, suggesting that dietary intake of plant sterols exhibit important biological activities in brain, some of which are critically involved in neurodegenerative disease pathogenesis, and might be useful bioactive food compounds to prevent AD. Several of these and other MOAs resemble our previous findings on DHA as well (see above). Beside directly increasing β- and γ-secretase activity, cholesterol significantly elevated gene expression of all γ-secretase components and of β-secretase BACE1, attended by an increase of PS1 and BACE1 protein level. Cholesterol increases Aβ generation by directly activating β- and γ-secretase enzyme activity and by elevating gene expression of β- and γ-

secretase. For β-sitosterol we observed a nearly identical increase in β- and γ-secretase activity as obtained for cholesterol resulting in significantly increased Aβ levels. However, in contrast to cholesterol, β-sitosterol only elevates gene expression of BACE1 and the corresponding protein level, but not of the γ-secretase components. This is in line with the observed lower increase in the Aβ level compared to cholesterol. In contrast to the amyloidogenic processing which is increased for cholesterol and β-sitosterol, non-amyloidogenic processing is affected differently by cholesterol and β-sitosterol. Cholesterol incubation resulted in significantly reduced α-secretase activity and sAPPα level. In contrast, β-sitosterol significantly increased α-secretase activity and sAPPα level, indicating that β-sitosterol activates amyloidogenic and non-amyloidogenic APP processing. The anti-amyloidogenic plant sterol stigmasterol revealed not significantly changed α-secretase activity and sAPPα secretion.
Based on these findings, cholesterol is highly amyloidogenic, whereas all analysed plant sterols are not as amyloidogenic as cholesterol to the point of stigmasterol which significantly decreased amyloidogenic processing of APP, emphasizing that plant sterols might be useful in AD prevention. Based on our *in vitro* findings, stigmasterol might be the most potent plant sterol for Aβ reduction.
In summary, our data on show that plant sterols exhibit striking similarity in terms of their MOA in amyloidogenic processing as compared to DHA, and might be useful bioactive food compounds which affect cellular functions that are known to be critically involved in neurodegenerative disease pathogenesis.

Through further clarification of the **molecular pathways** we identified that APP processing regulates ganglioside *de novo* synthesis and is affected in AD. We studied the regulation of the enzyme glucosylceramide synthase (**GCS**) which catalyzes the first irreversible step towards ganglioside biosynthesis, and the effect of ganglioside precursors. PS mediated APP cleavage down regulated GCS activity and gangliosides; resulting in a regulatory feed-back cycle linking APP processing with ganglioside biosynthesis. In the pathological situation of AD causing PS mutations, this regulatory cycle became defective *in vitro* and in an AD mouse model causing increased GCS activity and gangliosides. Notably, in sporadic AD brains GCS was increased as well. This indicates GCS as a target to lower Aβ production, but our subsequent analysis revealed that this omits an unexpected beneficial effect of the ganglioside precursors. Analysis of ganglioside precursors revealed strongly increased Aβ degradation mediated by the insulin-degrading-enzyme, an enzymes controlling brain Aß homeostasis. These ganglioside precursors are metabolized GM3-synthase (GM3S). Targeting GM3S combined the benefits of GCS inhibition and accumulation of ganglioside precursors, resulting in effective Aß lowering. Our study shows that GM3S could represent a novel target for AD therapy. Notably, a small reduction in GM3S is sufficient to effectively reduce Aβ levels.
Further analysis of gangliosides revealed that Aß and AICD regulate the activity of the GD3S (**ganglioside GD3-synthase**). This enzyme is important, because it controls the homeostasis of the two major lines of the major brain gangliosides.
Several APP cleavage products tightly regulate GD3-synthase (GD3S), the key enzyme converting *a*- to *b*-series gangliosides, and therefore controlling the level of the major brain gangliosides. Aβ peptides bind to the GD3S substrate GM3, thus reducing GD3S activity by substrate depletion, whereas the APP intracellular domain (AICD) downregulates GD3S transcription. *Vice versa,* GM3 decreases, whereas GD3, the GD3S product, increases amyloidogenic APP processing, resulting in a regulatory feedback cycle. Familial AD mutations increase GD3S activity and the expression of the GD3S is elevated in AD brains. Serial binding experiments showed that Aß and GD3S exhibit binding to each other only if Aß and GM3 are present already bound to each other. In such a case Aß presents the GD3S substrate GM3 in a manner that drastically reduces the turnover of GM3 to GD3 (the GD3S product). This is in agreement with findings that Aß has lipoprotein-like features and our previous results that Aß directly interacts with several different lipid-metabolic enzymes and that it activates some enzymes, whereas it inhibits others.
Like for SPT we found a strong regulation of **AGPS**, a key enzyme of **plasmalogen** synthesis, by AICD. Plasmalogens present a potential therapeutic target because plasmalogens are reduced early in AD pathogenesis, important for DHA mediated inhibition of Aß generation and the anti-oxidative potential of the brain.

Trans-fatty acids. Trans-fatty acids (TFA) are typical additions to food in the modern civilization and are a risk factor for coronary heart disease.
Hydrogenation of oils and diary products of ruminant animals leads to an increasing amount of trans-fatty acids in the human diet. Trans-fatty acids are incorporated in several lipids and accumulate in the membrane of cells. Here we systematically investigated whether the regulated intramembrane proteolysis of the amyloid precursor protein (APP) is affected by trans-fatty acids compared to the cis conformation. Our experiments clearly showed that trans-fatty acids compared to cis fatty acids increase amyloidogenic and decrease non-amyloidogenic processing of APP, resulting in an increased production of Aβ peptides, main components of senile plaques, which are a characteristic neuropathological hallmark for Alzheimer's disease. Moreover, our results show that oligomerization and aggregation of Aβ are increased by trans-fatty acids. The mechanisms identified by this *in vitro* study suggest that the intake of trans-fatty acids potentially increases the AD risk or causes an earlier onset of the disease. Although the increase in Aß is not as extreme as for example the one we observed with some gangliosides, this increase is still remarkable, because TFA are very well incorporated and end up in the brain. In contrast dietary gangliosides and most other Aß increasing lipids do not reach the brain in significant amounts.

DHA/lipid combinations within the molecule. It was speculated that the lipid composition/molecular structure in which DHA is present in the cell may strongly affect the biological activity of DHA. To test this, the common phospholipid and plasmalogen backbone/headgroups in presence of DHA were compared. Plasmalogens are especially suitable for this because they are strongly enriched in neurons, are a major sink/source for neuronal DHA and plasmalogens are strongly affected early on during AD etiology. Based on the molecular structure they allow for comparison with the respective ester bonded phospholipids which are other major neuronal lipids. There are indications, therefore, that the lipids are relevant to AD and that any observed effects may indeed be present, because these lipids constitute actual major neuronal lipids. All tested plasmalogen species showed a reduction in γ-secretase activity as compared to the respective ester lipid. FA composition affected the outcome and DHA was the most effective FA. Change from PE to PC strongly increased the Aß-lowering effect. ß- and α-secretase activity remained mainly unchanged, but for DHA a significant increase of α-secretase activity was observed. Plasmalogens were also able to decrease γ-secretase activity in human postmortem AD brains emphasizing the impact of plasmalogens in AD.

From brain to food. Analysis of phosphatidylcholines, lyso-phosphatidylcholines and phosphatidylcholin–plasmalogens derivates in Alzheimer's disease human post mortem brains and AD mouse model mass spectrometry lipidomics. Here a different approach was used to identify which fatty acids are of relevance for combination in a dietary approach. We screened patients with AD, healthy control subjects, AD transgenic mouse and mouse control brains were screened for dietary uptake of fatty acids. This allowed identification of possible disease relevant fatty acids and lipids suitable for dietary approaches. In particular the PC-lipidomics approach was able to identify several metabolites which were reduced as a consequence of AD pathology. Total PC and PUFAS, especially FA with 4 to 6 double bonds, are decreased in AD patients and transgenic mice models. In line with literature we observed a decrease in lyso-PC and PC-plasmalogens. In addition this approach allowed identification of FA candidates that are selectively changed in the AD brains and are significantly decreased both in PCaa and PC-plasmalogens. This finding suggests that the intake of PUFAs and elevation in the uptake of lecithin or increase in the Kennedy cycle, which is responsible for PC synthesis, might be beneficial in Alzheimer´s disease.

DHA alone is not **toxic for neuronal and endothelial** cells up to relative high concentrations.
The intake of the polyunsaturated fatty acid DHA has been associated with decreased amyloid deposition and reduced risk in AD in several epidemiological trials; however the exact underlying molecular mechanism remains to be elucidated. One aim of the study was to test whether DHA can exert a direct protective effect on the elements of the neurovascular unit, such as neurons, glial cells, brain endothelial cells, and pericytes, treated with **Aß42**. A dose-dependent high cellular toxicity was found in viability assays in all cell types and on acute hippocampal slices after treatment with Aß42 small oligomers prepared in situ from an isopeptide precursor. The cell morphology also changed dramatically in all cell types. In brain endothelial cells, damaged barrier function and increased para- and transcellular permeability were observed after peptide treatment. The production of **reactive oxygen species** was elevated in pericytes and endothelial and glial cells.

DHA significantly decreased the Aß42-induced toxic effects in all cell types measured by viability assays, and protected the barrier integrity and functions of brain endothelial cells. DHA also decreased the elevated rhodamine 123 accumulation in brain endothelial cells pre-treated with Aß42 indicating an effect on efflux pump activity. According to our experiments with an artificial Blood Brain Barrier (**BBB)** model the DHA significantly decreased the $A\beta^{42}$ induced toxic effect in all cell types of the neurovascular unit (endothelial cells, pericytes, gial cells and neurons). It is very likely a consequence of the general membrane stabilizing effect of DHA and it is in connection with the special DHA conformation in lipid bilayers. Pretreatment of endothelial cells with DHA provides almost full protection against the toxic effect of Aß 1-42. DHA may be a potent agent to prevent $A\beta_{42}$ induced damages on the elements of the neurovascular unit acting via multiple ways. Based on these new observations DHA might exert a protective effect not only on neurons but also on the BBB and its functions, and this double effect may be beneficial in AD. DHA shows a general membrane stabilizing effect in all brain cells, based on its special conformation and thus may protect all the brain cell types.

These results indicated for the first time that DHA can protect not only neurons but also the other elements of the neurovascular unit from the toxic effects of Aß42 and this effect may be beneficial in AD.

Muscarinic transmission. A role of muscarinic transmission in the clinical picture of the disease is generally recognized. In fact, the majority of FDA approved drugs for AD target the cholinergic system. However, the mechanism(s) underlying deterioration of the cholinergic system in AD and the expected beneficial role of dietary supplements in relieving disease progression are not clear. It has previously been revealed that there is progressive impairment of muscarinic transmission in brains of APPswe/PS1dE9 transgenic female mice between the age of 7 and 17 months. The main goal of this piece of the LIPIDIDIET project was to ascertain what aspects of cholinergic transmission are targeted by accumulating Aβ fragments and what is the influence and mode of action of various lipidic and non-lipidic dietary supplements, and their combinations, on cholinergic transmission. We demonstrate that the impairment of brain cholinergic transmission develops in parallel with the increase of soluble Aβ1-42 and mainly afflicts transmission mediated by the M1 receptor subtype. Next it was shown that various combinations of lipidic and nonlipidic nutritional components exhibit profitable effects on muscarinic transmission both in cell lines and in a transgenic mouse model of AD that is mainly due to recovery of receptor/G-protein coupling.

In order to determine whether cholinergic impairment correlates with amyloid pathology or the increase in soluble Aβ fragments, young transgenic mice that do not yet show amyloid pathology and adult transgenic mice that just start to form amyloid plaques were used. Despite already detectable increase in tissue concentration of soluble human Aβ fragments 1-40 and 1-42 in the brain there were no differences in markers of cholinergic synapses, muscarinic receptor-mediated G-protein activation, and acetylcholine release from cerebral slices ex vivo between young transgenic and littermate wild type mice. In 5-6 month-old transgenic mice the evoked release of preformed acetylcholine (ACh) measured in brain slices either increased (cortex) or decreased (hippocampus, striatum). These differences are most likely due to adaptive changes that might be related to region-specific rate of amyloid accumulation. Potency of the muscarinic agonist carbachol in activating G-proteins in cortical membranes already demonstrates significant decrease. These data evidence postsynaptic impairment of cholinergic muscarinic transmission that develops *in vivo* along with soluble β-amyloid accumulation that is faster for Aβ1-42, and most likely afflicts the M1 receptor subtype that is the major receptor subtype in brain. In addition, these results demonstrate incipient presynaptic impairment that may play a role during increased cholinergic activity. These results suggest that the accumulation of soluble Aß1-42 plays a major role in the impairment of muscarinic transmission. In *in vitro* experiments on Chinese hamster ovary cells that selectively express individual subtypes of muscarinic receptors (M1 through M5) we probed the expected influence of soluble Aß1-42 on individual subtypes of muscarinic receptors. 4-day treatment of cells with relatively low concentrations of Aß1-42 specifically changed the binding characteristics of carbachol at the M1 receptors. The treatment decreased affinity of high-affinity carbachol binding and increased the fraction of high affinity binding sites. The M1, M3 and M5 receptors preferentially couple with Gq/11 G-proteins while the M2 and M4 receptors with Gi/o G-proteins. These results thus point to a selective impairment of muscarinic receptor/Gq/11 G-protein coupling.

It has been demonstrated that Aß1-40 regulates cholesterol synthesis and that plasma membrane cholesterol concentration is important for proper APP processing. Membrane cholesterol level decreases during aging and in line with the established physiological role of amyloid β1–40 fragments in inhibiting cholesterol synthesis the reduction is even more manifest in brains of a subset of Alzheimer's patients. Increased membrane cholesterol reduced the maximal effect of M1 receptor stimulation but had no effect at M2 and M3 receptors. Reduced membrane cholesterol strongly inhibited maximal response of the M1 and M3 receptor stimulation but augmented that of the M2 receptor stimulation. These results show that the most sensitive is signaling through the M1 subtype that is negatively influenced by both the increase and decrease in membrane cholestrol. In this context it is worth to note that the M1 muscarinic receptor subtype is not only involved in cognitive functions but also participates in non-amyloidogenic cleavage of APP.

Inflammation. Epidemiological studies suggest that diets enriched with omega-3 fatty acids, e.g. DHA, reduce risk for Alzheimer's disease. However, the underlying anti-AD mechanism remains unclear. In AD, microglial activation serves as a double-edged sword: on one side, they injure neurons by releasing cytotoxic inflammatory molecules; on the other side, they protect neurons by clearing pathogenic Aβ. In this study, it was observed that DHA reduced Aβ aggregates-induced both protein secretion and gene transcription of inflammatory cytokines (e.g. TNF-α and IL-6) and chemokines (e.g. CCL-2) in microglia/macrophages. It was further demonstrated that DHA suppresses not only TLR 2- and TLR4-, but also TLR3- and TLR9-, and even interferon-γ-induced inflammatory activation in primary macrophages. Moreover, the study showed that DHA does not impair the Aβ internalization by macrophages. Thus, omega-3 fatty acids inhibit Aβ-triggered microglial neurotoxic inflammatory activation, but keep microglial beneficial effect to clear Aβ. This study provides one more line of evidence for the anti-AD effect of omega-3 fatty acids in AD patients.

Many studies have demonstrated that Aβ aggregates can injure neurons directly and indirectly via induction of microglia/brain macrophages-dominated chronic inflammation. The use of non-steroidal anti-inflammatory drugs was associated with a reduced risk for later AD. Epidemiological studies showed that diets supplemented with omega-3 polyunsaturated fatty acids (PUFAs), e.g. DHA, can modulate inflammatory profiles in humans. In cultured macrophages, omega-3 PUFAs were observed to inhibit TLR2 and TLR4-induced inflammatory activation. Omega-3 PUFA-enriched food was associated with the reduced risk of AD, especially when sufficient PUFAs are taken before development of clinical dementia, although DHA-supplemented diet was also reported not to benefit mild to moderate AD patients. In Aβ precursor protein (APP) transgenic mouse models, similar “anti-AD” effects of DHA-supplemented diet were observed; these effects include improved cognitive deficits, reduced Aβ deposition, and decreased phosphorylated tau inside of neurons. Thus, the effects of PUFAs on AD pathophysiology and the related potential mechanisms need to be investigated.
In LIPIDIDIET, the hypothesis that omega-3 PUFAs suppress Aβ-induced neurotoxic inflammatory activation was tested and the potential mechanisms further investigated. **DHA reduces non pro-inflammatory cytokine secretion in macrophages.** Since innate immune receptors are key receptors for microglia/macrophages to recognize dangerous signals in- or out-side of the brain and CD14, Toll-like receptor TLR2 and TLR4 have been reported to recognize aggregated Aβ and mediate inflammatory responses, we investigated how DHA modulates TLR2- and TLR4-initiated inflammatory activation. DHA treatment significantly reduced Pam3CSK4 (TLR2 ligand) and LPS (TLR4 ligand)-induced TNF-α secretion from bone marrow-derived macrophages (BMDMs) in a concentration-dependent manner. Similarly, the secretion of IL-6 was significantly decreased by DHA. We further investigated the modulatory effects of DHA on other TLR-initiated inflammatory activation. DHA markedly suppressed Poly I: C (TLR3 ligand) and CpG ODN (TLR9 ligand)-induced TNF-α and IL-6 secretion.
In order to exclude the possibility that the DHA suppresses inflammatory activation via inducing **cell death**, the activity of caspase 3 (a marker for apoptosis) and the release of lactate dehydrogenase (LDH, to detect loss of cell integrity) from macrophages following the DHA treatment was analysed. No significant cell death caused by DHA was observed. IFN-γ is an important endogenous inflammatory activator and stimulates different signaling through the pathways other than TLRs. Thus, the effects of DHA on **IFN-γ-initiated inflammatory activation** were tested. Secretion of IP-10 was significantly decreased by DHA. The suppressive effect of DHA was independent of MyD88. Following our investigation of general anti-inflammatory effects

of DHA, we examined the effects of DHA on **Aß induced inflammatory activation**. We observed that DHA treatment significantly reduced Aß42 aggregate-induced TNF-α secretion from macrophages. Similarly, Aß42 aggregate-initiated IL-6 secretion was also significantly decreased by DHA. DHA did not reduce Aß42 aggregates-initiated IL-10 secretion. AA significantly increased Aβ-initiated IL-6 secretion. We observed that DHA decreased Aβ-induced PGE2 release in BMDMs, AA markedly enhanced PGE2 secretion. After we have observed that DHA suppressed Aβ-induced inflammatory activation, we investigate whether the same inhibitory effects of DHA existed in **microglia**. Microglia pretreated with DHA and then treated with oligomeric Aβ42. **DHA pretreatment** significantly reduced Aβ-induced release of TNF-α and CCL-2. The inflammatory activation has been divided into **pro- and anti-inflammatory activation**. We isolated the total RNA from the Aβ42-activated microglia with or without DHA co-treatment. Aβ42 significantly increased the pro-inflammatory gene and the *IL-10* transcription. This activation was inhibited by 25μM DHA. After we have observed that DHA inhibits the inflammatory activation in both microglia and macrophages, we continued to investigate the potential mechanism. TACE (ADAM17) is the enzyme to convert pro-TNF-α to active TNF-α, thus, serves as a key player in **microglial inflammatory activation**. DHA treatment significantly reduced microglial TACE activity with and without inflammatory activation by TLR2 ligand, Pam3Cys-SKKKK. However, DHA treatment changed the expression of neither TACE nor ADAM10, which has similar enzymatic activity of ADAM17. DHA potentially affects the cell membrane components and reconstructs the membrane structure, which leads to the **lipid raft** reorganization and the clustering of receptors (like TLR2 and TLR4) on the raft. Thus, we investigated whether DHA modifies TLR2-induced lipid raft reorganization in macrophages. We first compared the distribution of flotillin in the sequential fractions derived from the THP-1 cells stimulated with or without Pam3Cys-SKKKK. Flotillin shifted from fraction 5 to fraction 4 after TLR2 activation. However, after pre-treatment with DHA, the shifting of flotillin did not change. Aβ was shown to directly stimulate microglia to secrete **reactive oxygen species** (ROS) which also damage surrounding neurons. Microglia were treated with Aβ and observed that Aβ increased the release of ROS. Pretreatment with DHA decreased the release of ROS induced by Aβ. Oxidative stress is an important component in the inflammation. DHA treatment showed that HO-1, but not Nrf2 was significantly up-regulated. Nrf2 is a leucine zipper transcription factor, which is stimulated by various conditions of oxidative stress and plays a key role in the transcriptional activation of several antioxidant-responsive elements (ARE)-mediated genes, including *ho-1*. We compared the DHA-caused reduction of TNF-α and CCL-2 amount after Aβ activation between groups with and without pre-treatment of ZnPP. ZnPP had no effects on the DHA-induced reduction of inflammatory secretion. Growing evidence has suggested that microglial elimination of Aβ protects neurons against AD-related neurodegeneration. We tested the effect of DHA on **Aβ internalization by macrophages**. Internalization of Aβ42 aggregates was not altered by DHA or EPA or AA. DHA treatment increased transcription of *RAGE* and *CD36*; although the transcription of *SR-A* and *CD14* was decreased in the DHA-treated cells. Thus, DHA did not reduce the Aβ phagocytotic capacity overall. Ginkgo extract with DHA reduced microglial inflammatory activation by enhancing autophagy-mediated inflammasome degradation.

For inflammation it can be concluded that besides the effects of PUFAs to reduce Aβ production, it was observed that DHA and EPA inhibited Aβ-induced secretion of proinflammatory cytokines, TNF-α and IL-6, and chemokine CCL-2, but not antiinflammatory IL-10 from both microglia and macrophages. Furthermore, DHA was demonstrated to suppress the transcription of proinflammatory genes, such as TNF-α, CCL-2 and iNOS. Thus, our results support that PUFAs-supplemented functional diets reduce neurotoxic inflammatory activation in AD patients.

Mitochondrial dysfunction. Mitochondrial dysfunction is an established phenomenon in Alzheimer's disease, but the causes and role of PS1, APP and APP's cleavage products in this process are largely unknown. The effect of these AD-associated molecules on mitochondrial features was studied. PS1 was found to affect mitochondrial energy metabolism (ATP levels and oxygen consumption) and expression of mitochondrial proteins. These effects were associated with enhanced expression of the mitochondrial master transcriptional coactivator PGC-1α. This protein is a master regulator of mitochondrial energy metabolism and is strongly linked to physical activity in health and disease. Importantly, PS1-FAD mutations decreased PS1's ability to enhance PGC-1α mRNA levels. Analyzing the effect of APP and its γ-secretase-derived cleavage products Aβ and AICD on PGC-1α expression showed that APP and AICD increase PGC-1α expression.

Accordingly, PGC-1α mRNA levels in cells deficient in APP/APLP2 or expressing APP lacking its last 15 amino acids, were lower than in control cells and treatment with AICD, but not with Aβ, enhanced PGC-1α mRNA levels in these cells. Importantly, APP/AICD increase PGC-1α expression also in the mouse brain. Our results therefore suggest that APP processing regulates mitochondrial function and that impairments in the newly discovered PS1/APP/AICD/PGC-1α pathway may lead to mitochondrial dysfunction and neurodegeneration.
The lipoprotein apoE is the main genetic risk factor for AD. We found that DHA affects lipidation in apoE dependent manner identifying a molecular basis for the previously observed differential response by apoE3/4 carriers.
Further mechanisms were identified, including the lipid dependent localization of the Amyloid precursor protein, impact of DHA oxidation on amyloidogenicity, AICD signalling, DHA impact on endothelial function and ROS, apoE lipidation and receptors.
These examples not only illustrate how successful this WP had been thus far, but also how important it is to not only focus on isolated effects, but rather to study the role of DHA in neuroprotection as a whole in a comprehensive manner. Last but not least this WP has also generated some very important input for WP4 and strongly cooperates with the animal WPs 5-7 to validate the effects discovered here for *in vivo* relevance as much as it receives input and ideas to follow-up from those WPs.

ApoE. Accumulation and aggregation of Aß Activation of the amyloid cascade by inhibition of the Aβ degrading enzyme neprilyisn results in isoform specific degeneration of hippocampal CA1 neurons of apoE4 but not of the corresponding apoE3 mice. The extent to which this neurodegeneration is associated with the accumulation of intracellular Aβ and oligomerized Aβ was investigated. This was performed by immunohistochemical and immunoblot experiments. Accordingly, double labeling confocal microscopy utilizing anti-Aβ42 and a mAb directed against the lysosomal marker cathepsin D (CatD) revealed that Aβ co-localized with lysosomes and does so preferentially with the large ones. Furthermore, the kinetics of accumulation of "free-Aβ" and of the Aβ which co-localized with the large lysosomes were similar. Importantly, these effects are specific to the treated apoE4 mice and are not observed in the corresponding apoE3 mice. Additional analysis revealed that at the plateau about 15% of the accumulated Aβ and 20% of the lysosomes co-localize. The extent to which the Aβ which accumulates in the affected neurons oligomerizes was next examined utilizing mAb I-11 which is directed specifically at the oligomeric backbone of amyloid. This was pursued by double labeling confocal microscopy utilizing I-11 and anti-Aβ42 mAbs. Quantization of the results obtained revealed that ~15% of the total I-11 and ~15% of the Aβ42 positive staining co-localize. This population will be referred to as oligomerized –Aβ (oligo-Aβ). Importantly the oligo-Aβ, like the total Aβ, co-localizes preferentially with large lysosomes. The results show that the neuropathological effects of apoE4 and Aβ are associated with the accumulation of intracellular Aβ which is followed by its oligomerization. This suggests that the neuropathological impairments and the resulting behavioral deficits which are induced by apoE4 are driven by the accumulation of oligomerized Aβ in the affected neurons.

The effects of apoE4 on the levels of apoE receptors. These experiments focus on the metabolism of apoE and on assessing the possibility that the lipidation of apoE and the levels of expression of distinct apoE receptors are modulated isoform specifically by apoE4 and thus may play a role in mediating the pathological effects of apoE4. The results obtained reveal that ApoE4 has marked effects on the lipidation of apoE and on the levels of its receptor. This is manifested by hypolipidation of apoE4 and by the specific down regulation of the apoER2 receptor.
Direct interactions between apoE and Aβ We examined whether apoE4, Aβ and oligo-Aβ, co-accumulate in the same organelles following activation of the amyloid cascade. ApoE, Aβ and oligo-Aβ accumulated in the same sub group of large lysosomes of the apoE4 mice following activation of the amyloid cascade. Thus, suggesting that these molecules interact within the activated lysosomes which are formed following activation of the amyloid cascade.

Determination of the intracellular neuroprotective site of action of DHA. SorLA/LR11 is an intracellular receptor which plays a role in the transport of molecules engaged in numerous intra cellular processes such as endocytosis and signal transduction. The levels of SorLA decrease in AD and it has been suggested that DHA can affect the life cycle of SorLA. Immunoblot experiments revealed that SorLA is composed of a MW 250KD band whose levels were similar in apoE4 and apoE3 mice maintained on the control diet. Exposure to the DHA diet markedly increased the levels of SorLA of the apoE3 mice but had no effect on the corresponding apoE3

mice**.** These findings are consistent with the hypothesis that when exposed to DHA, apoE3 but not apoE4 interacts isoform specifically with SorLA.

Reversal of the the hypolipidation of apoE4 by increasing the RXR agonist bexarotene. Treatment with the RXR agonist bexarotene markedly increased the extent of lipidation of apoE4 *in vivo* without affecting the total levels of apoE4. Additional experiments revealed that bexarotene increases the levels of the lipidation proteins ABCA1 and ABCG1.

Regarding the MOA, it can be concluded that DHA interacts with multiple mechanisms involved in neurodegeneration in general and, especially, in AD. Indeed the means of DHA activity appears to build on pleiotropic mechanisms which only taken together explain the overall results. Importantly, no potentially negative impact of DHA was observed.

WP4 - Enhancing the effectiveness of nutritional formulations on neuroprotection

The main objective of this WP is to develop more effective lipid-based nutritional compositions. This main objective breaks down into 3 sub-objectives (tasks) which are**:**

1) to identify the lipid related factors which increase the effectiveness of DHA-based nutrition on neuroprotection
2) to evaluate the dietary fat blends and individual lipids or FA for their interaction with effect reducing lipids
3) to use the information on the MOA of DHA to develop more effective nutritional compositions

Nothing was known in the published literature of how to increase the effectiveness of DHA-based nutrition on neuroprotection. Current nutritional products containing DHA mainly differ in their DHA and EPA content. To date, there are three general approaches to enhance the synaptic stimulation of DHA: (1) Precursors - *In vivo* DHA consumption results in mild increases in synaptic and neurite fibrillar proteins. However, if DHA is consumed together with metabolic precursors like choline and a pyrimidine source (e.g. uridine), moderate to strong effects on synaptic proteins and synapse formation are achieved (Wurtman et al. 2006). (2) Combination approach - Another way to increase the effectiveness of fatty acid is to combine them with different lipids. (3) MOA exploitation - In WP3 we are investigating the effective target for DHA interaction in neuroprotection. Because of the nature of DHA it is reasonable to assume that this target(s) may be addressable by other lipids or DHA metabolites. Here the information obtained in WP3 is exploited to further enhance the effectiveness of the lipid-based nutritional composition. Outcomes of this WP are be forwarded to WP2 for diet generation and WP's 5 –7 for *in vivo* evaluation (mouse).

Several lipidic factors could be identified which can either decrease or increase the effectiveness of DHA. Importantly, some choline shows significant neuroprotective effect against Aß1-42 oligomers together with DHA on the acute hippocampal slices, without any toxic effects) can strongly increase this effectiveness, whereas the individual components have virtually no neuroprotective properties. DHA significantly decreased the Aß42 induced toxic effects in all cell types of neurovascular unit, measured by viability assays, and protected the blood-brain-barrier integrity. Regeneration capabilities of vascular endothelial HUVEC cells were studied. Combined supplementation of omega-3 PUFAs, phospholipids, and B-vitamins enhanced endothelial cell migration more that single-nutrient supplementation. It was also found that low concentrations of soluble forms of amyloidogenic fragment Aß (1-42) that do not yet demonstrate overt toxicity already impair muscarinic receptor-mediated signal transduction, specifically through muscarinic M1 receptor. It demonstrates that disturbance of the muscarinic neurotransmission develop rather early, during the prodromal (silent) stage in the pathogenesis of Alzheimer´s disease.

Further it was found that such combinatory formulations can still be further increased in their effectiveness, by addition of other nutritional factors. E.g. shorter fatty acids, ineffective for γ– and ß-secretase, have a strong impact on α-secretase activity. At the same combinations including lipidic (e.g. certain fatty acids) and non-lipidic nutritional factors (e.g. it was also observed that some molecules can revert or modify the neuroprotective capacity and the same fatty acid composition, but altered conformation (cis – versus trans fatty acids) significantly changes the amyloidogenic potential of the respective lipids. Therefore careful selection and intense validation *in vitro* as well as *in vivo* is essential before reliable conclusions can be drawn. One example of this is the identification of stigmasterol and the additive effects in combination with DHA. Whereas

WP4 performs some pilot evaluations *in vivo* to verify e.g. effective uptake and cerebral efficacy, long term studies and in depth *in vivo* analysis is done by WPs 5 – 7.

Choline. Choline shows significant neuroprotective effect against Aß1-42 oligomers together with DHA on the acute hippocampal slices, without any toxic effects. Pyrimidine does not show similar effects. The neurotoxic effect of Aβ and the neuroprotective effect of DHA, choline and pyrimidine was studied. Differentiated SH-SY5Y neuroblastoma cells as well as rat hippocampal slices were used measuring cell viability with MTT assay. DHA and choline shows partial protection of the neuronal and glial cells in concentration, pyrimidine treatment was ineffective. DHA plus choline treatment showed 90% protection against the neurotoxic effect of Aβ. Pyrimidine showed no synergistic effect giving together with DHA. Choline has no toxic effect on the acute hippocampal slices. These results demonstrated the synergistic effect of choline on the neuroprotection of DHA against Aβ1-42 and supported the hypothesis that combinations of agents may result in more effective nutritional compositions.

UMP. *In vivo* studies (rats fed with DHA UMP and vitamin-B enriched diet) demonstrate that DHA in combination with UMP causes significant reduction of both β- and γ-secretase activity. The brain DHA-level of the rats after DHA/EPA/UMP diet is elevated by approximately 12% compared to the control group.

Vitamin D. 90% of the elderly population has a vitamin D hypovitaminosis and several lines of evidence suggest that there might be a potential causal link between Alzheimer´s disease and an insufficient supply of vitamin D. However the mechanisms linking AD to vitamin D have not been completely understood. The aim of this study is to elucidate the impact of 25(OH)-vitamin D3 on APP processing in mice and N2A cells utilizing very moderate and physiological vitamin D hypovitaminosis in the range of 20-30% compared to wt mice. It was found that already under such mild conditions Aß is significantly increased, which is caused by increased ß-secretase activity and BACE1 protein level. Additionally, neprilysin (NEP) expression is down-regulated resulting in a decreased NEP activity further enhancing the effect of decreased vitamin D on the Aß level. In line with the *in vivo* findings, corresponding effects were found with N2A cells supplemented with 25(OH)-vitamin D3. These results further strengthen the link between AD and vitamin D3 and suggest that supplementation of vitamin D3 might have a beneficial effect in AD risk reduction.

To investigate presumed effects of different lipids and their combinations with non-lipidic components on **receptor-mediated signaling** the mouse neuroblastoma cell line N1E-115-1 that naturally expresses muscarinic receptors was used. Cells were grown in the presence of different supplements and their combinations. It was found that lipidic supplements (DHA, EPA, α-tocopherol) significantly reduced activity of the executive cell death enzyme caspase-3 to a similar extent. Furthermore, their combinations exhibit an additive effect.

The short-term and long-term effects of selected diets on muscarinic receptor-mediated signal transduction and markers of cholinergic synapses in brains of APPswe/PS1dE9 transgenic female mice was investigated.

In the short-term experiment, mice were fed tested diets denoted Fortasyn, Sterol, and Sterol+DHA ad libitum. Hippocampi were used for biochemical determinations. Transgenic mice exhibited reduced potency of carbachol in stimulating GTP-γ35S binding compared to wild type mice. Unlike the decline in the density of muscarinic receptors, activities of choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and butyrylcholinesterase (BuChE) were higher. Transgenic animals where all tested diets significantly increased potency of carbachol in stimulating GTP-γ35S binding up to values obtained in wild type mice. In addition, Fortasyn significantly increased activity of ChAT, AChE and BuChE.

In the long-term experiment, mice were fed tested diets denoted Combination (Combi), Endothelial (Endo), and Mitochondrial (Mito) ad libitum for seven months. Endothelial diet contained fish oil and vascular module components, Mito diet fish oil and energy module, and Combi diet was a mixture of Fortasyn and Endothelial diets. In comparison with wild type mice fed control diet transgenic mice demonstrated a decrease in ChAT and BuChE activity, and the decrease in potency and maximal effect of carbachol in stimulating of GTP-γ35S binding in frontal cortex the. In the hippocampus, the density of muscarinic receptors and ChAT acivity was higher, and the potency, but not efficacy, of carbachol in stimulating GTP-γ35S binding was reduced. In contrast,

expression of presynaptic genes for ChAT, vesicular acetylcholine transporter (VAChT), and cholinergic high affinity choline transporter (ChT) was reduced in hippocampus of transgenic mice.

In summary, these experiments on receptor-mediated signaling show that lipidic supplements DHA, EPA, and α-tocopherol demonstrate general protective effect that is additive while tested nonlipidic supplements have no effect. These observations support the notion that combinations of the tested supplements offer preferable protective effect than DHA alone that serves as the established standard treatment and indicate that the particular combinations of nutrients demonstrate selective effect on various aspects of cell metabolism Taken together, these results demonstrate that different lipid-based diets have differential profitable effects on signal transduction that does not necessarily depend on the decrease in Aß production and supports the viability of a nutritional approach in the treatment of AD.

Effects of individual classes of nutrients in the membrane potential assay

The effects of supplementation with individual classes of nutrients on Carbachol-induced changes. in membrane potential in PC12 cells are depicted in Fig. 1. Components were selected from groups of precursors and combinatory agents, i.e. nutrients reported to act synergistically with DHA on MOAs relevant to membrane composition, synaptic functioning, cholinergic neurotransmission, and neuroprotection. The data indicate that, individually, only the omega-3 PUFAs DHA and EPA induced a significant increase in muscarinic receptor activation induced by Carbachol.

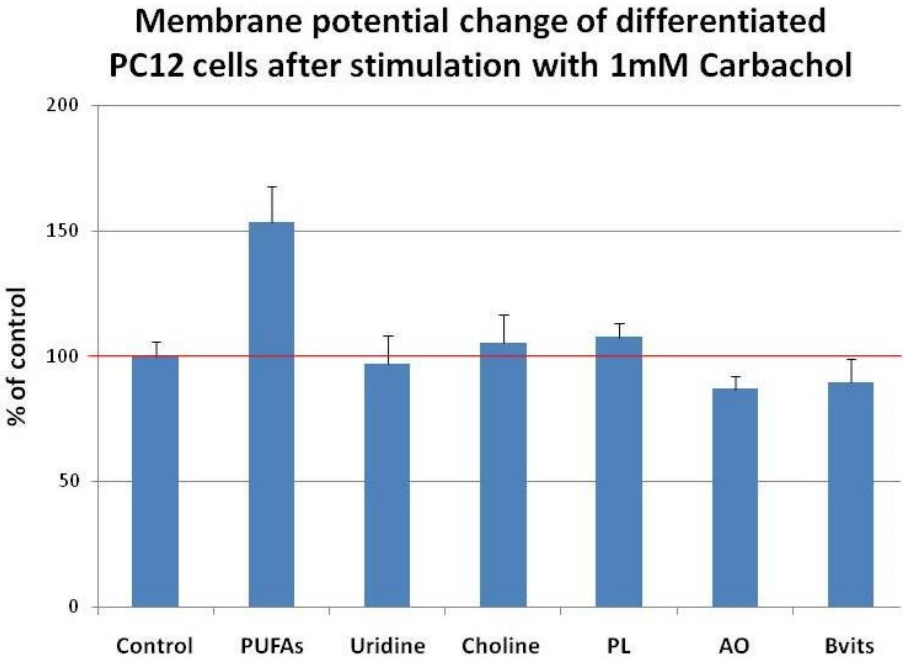

Fig. 1: *Effects of n3-PUFAs, uridine, choline, phospholipids, antioxidants, and B-vitamins on Carbachol-induced changes in membrane potential. Supplementation with PUFAs was found to enhance the effects of muscarinic receptor stimulation. Other classes of nutrients either induced a non-significant increase or a small decrease in membrane potential as compared to control, when given alone.*

Effects of combining classes of nutrients in the membrane potential assay

The effects of supplementation with combined classes of nutrients on Carbachol-induced changes in membrane potential in PC12 cells are depicted below in Fig.2. Combinations of these nutrients were found to act additionally and synergistically with PUFAs. The most optimal effects of supplementation were observed after combining all five classes of nutrients.

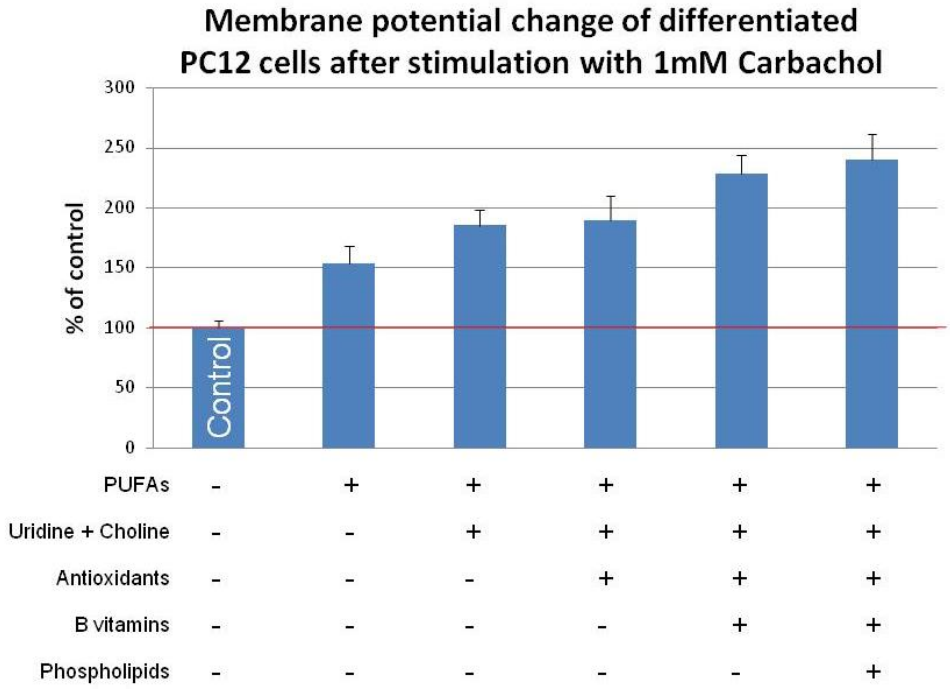

Fig. 2: *Effects of combining n3-PUFAs, uridine, choline, antioxidants, B-vitamins, and phospholipids on Carbachol-induced changes in membrane potential. Additional supplementations on top of the PUFAs were found to further enhance the effects of muscarinic receptor stimulation as compared to control.*

It can be concluded from this WP that evaluation of the current and previous results identified most effective factor combination contributing to the efficacy of DHA based nutrition regarding the essential AD-relevant factors under investigation. Over the course of the project it has become clear that, while DHA has positive effects on several mechanisms relevant for AD, the combined administration with nutrients like B-vitamins, uridine, choline, anti-oxidants, or phospholipids does significantly enhance the effects observed *in vitro*. Synergies and additive effects were confirmed in the *in vivo* studies with different cycles of rodent diets, where the further enrichment of nutritional concepts yielded similar or somewhat less positive results. Together these data suggest that a more effective lipid-based nutritional composition with a close to optimal neuroprotective profile has been developed.

WP5 - Animal model - ApoE

The objective of this WP is **to directly test the ability of diets to prevent, delay or reverse the brain pathological effects of apoE4**. ApoE ε4 is the major genetic risk factor for Alzheimer's disease, but it is not known how apoE4 increases the risk for dementia. Recent clinical trials for treatment showed that with carriers of apoE4 either more unwanted side effects or a reduced response was observed as compared to carriers of the non-risk apoE3 gene. It is therefore critical to evaluate the effect a potential treatment has on apoE4, even more so as one physiological function of apoE4 is to shuffle lipids between neurons and astrocytes. Since the interventions tested in LIPIDIDIET are primarily lipid based, a cross-talk to apoE3 or 4 is not unlikely. This study investigated the molecular mechanisms underlying the behavioural and brain pathological effects of apoE4, the most prevalent genetic risk factor of AD, and assessed the extent to which they can be prevented by diet.

The effects of the nutrition combinations were assessed utilizing several diets (DHA/fish oil; cholesterol; plant lipids; mitochondrial, endothelial, in addition the Fortasyn and a reference diet).

Hippocampal glutamatergic nerve terminals are affected by apoE4 and environmental stimulation in a diet dependent manner. Accordingly the isoform specific effects of apoE4, which are apparent in reference and high cholesterol diets are neutralized in fish oil and Fortasyn diets both of which are enriched in DHA. In contrast the effect of apoE4 was only partially reversed in the sterol diets (Fig.1).

The learning and memory impairments of apoE4 mice which were maintained on reference and high cholesterol diets were reversed in corresponding mice which were maintained on fish oil (DHA) diet. Importantly, apoE4 mice maintained on either the Fortasyn or the sterol diets, both of which contain fish oil, performed less well than corresponding mice which were fed the fish oil diet.

Reversal of behavior impairments of the apoE4 mice. These experiments revealed, utilizing the fear conditioning paradigm, that maintenance of the apoE4 mice on the DHA diet corrects the memory impairments which persist in such mice maintained on control diet. Furthermore this is also associated with improvement of the nest building behavior of the apoE4 mice which is erratic for apoE4 mice fed on the control diet.

The levels of Aβ in the hippocampus are affected by apoE4 and environmental stimulation in a diet dependent manner. Accordingly, these isoform specific effects of apopE4, which are apparent in reference and high cholesterol diets are neutralized in the fish oil and Fortasyn diets which are both enriched in DHA. In contrast the effects of apoE4 on hippocampal Ab were not reversed in a fish oil+ sterol diet (Fig.1).

Brain lipid metabolism: The level of cholesterol metabolism is lowered specifically by the Fortasyn diet and is not affected by the apoE4 genotype. ApoE4 decrease the levels of hippocampal phospholipids in cholesterol fed mice and this effect is prevented by the DHA diet and reversed in the Fortasyn and sterol diets.

Reversal of the brain pathological effects of apoE4. These experiments focused on general presynaptic markers such as the vesicular protein synaptophysin as well as on markers of specific neuronal systems including Vglut. Vgat and VACh which are specific to the presynaptic synaptic vesicles of the glutamatergic, GABAergic and cholinergic nerve terminal. These experiments revealed that all of the synaptic impairments which were observed in apoE4 mice maintained on control reference diet were reversed by the DHA diet and that this protective effect was apparent at least up to the age of 12months. Fig. 2 depicts such representative results which focus on the effects of the apoE genotype and diet on the levels of the glutamatergic presynaptic molecule Vglut. As can be seen, both the effects of apoE4 at 4 months and the genotype independent effects of aging, which were obtained in the regular diet, were abolished in mice maintained on the DHA diet.

In conclusion these experiments show that both DHA and Fortasyn diets can prevent appearance of apoE4 induced brain pathology and cognitive impairments and that this effect is maintained in young and 12 months old adult mice alike.

Testing the different novel diets in mice that carry either the apoE3 or the apoE4 allele, it was found that DHA counteracts the age dependent neuropathological effects of apoE4, that DHA counteracts the cognitive impairments of apoE4, that apoE4 is hypolipidated relative to apoE3, that the pathological effects can be reversed by increasing the lipidation of apoE4. The RXR agonist bexarotene was found to be able to reverse the lipidation deficiency of apoE4, cognitive impairments and the associated neuronal deficits of apoE4 mice. The effect of apoE4, the main genetic risk factor for Alzheimer's disease, on the levels of two apoE-receptors, apoer2 and Lrp1was investigated in different experimental paradigms. It was shown that naïve apoE4 mice have decreased levels of the apoE receptor, Apoer2, while activation of the amyloid cascade results in up-regulation of the apoE receptor Lrp1. This study demonstrated that the levels of hippocampal apoE receptors Lrp1 and Apoer2 *in vivo* are affected isoform specifically by apoE4 and that the type of receptor affected is context dependent. We developed a model to study the early onset pathological effects of apoE4. This model revealed that apoE4 induces cognitive deficits associated with neuronal and synaptic pathology.

It was also observed, however, that addition of some molecules can completely eradicate some effects or generate totally new one. Highlighting the fact that diet composition needs to be validated against certain risk factors, e.g. apoE.

From this, it can be concluded that the mechanistic studies revealed that apoE4 interacts synergistically with Aβ, which is a key pathological player in AD, and that this interaction is associated with impaired lipidation of apoE4. Furthermore correction of the lipidation impairments of apoE4 by distinct pharmacological strategies blocks and prevents the apoE4 driven behavioural and brain related impairments. Consistent with these results, it was shown that the effects of

apoE4 *in vivo* are markedly affected by the addition of distinct lipids to the diet and that DHA, which is an important constituent of fish oil, blocks and prevents the negative effects of apoE4 most effectively.

WP6 - Animal model - Amyloid

The main objective is **to directly test the efficacy of new lipid-based test diets to slow down amyloid accumulation and the development of memory impairment.** Amyloid, especially soluble toxic oligomers, are believed to cause Alzheimer's disease. This WP is expected to give the *in vivo* result of the effect of the diets on amyloid parameters. It does not contain as much mechanistic investigations, since those are done in other WPs. Analysis parameters are those which have proven to be the best indicators for neuroprotection. These are (soluble) Aß levels, Aß species, ratios and truncations and of course cognitive performance/ behaviour.

The objective was to assess the efficacy of new lipid-based test diets in preclinical mouse models in their ability to slow down amyloid accumulation and delay development of memory impairment with aging. Two sets of diets were tested: five experimental diets (later, Experiment 1) and three adjusted experimental diets (later, Experiment 2). Previous published data have suggested that dietary fish oil, providing n3 PUFAs like eicosapentaenoic acid (EPA) and DHA, are associated with reduced dementia risk in epidemiological studies and reduced amyloid accumulation in mouse models of Alzheimer's disease (AD). In LIPIDIDIET it was studied whether additional nutrients can improve the efficacy of fish oil in alleviating cognitive deficits and amyloid pathology in amyloid plaque producing transgenic and wild-type mice. Transgenic mice carrying familiar AD-linked amyloid precursor protein (APPswe) and presenilin-1 (PS1dE9) mutations were used as study subjects. These mice are referred to as APP/PS1 mice. Female mice were used in all experiments, since AD is more common in women than in men. Conversely, the Nijmegen group conducted parallel experiments in male mice of the same line. Both experiments comprised 70 APP/PS1 mice and 20 wild-type (WT) littermates.

Experiment 1 compared four isocaloric (5 % w fat) diets: **Control:** the basis for all experimental diets. **Fish oil**: fish oil (3% of total fat) substituted soy oil of the Control diet. **Fortasyn**: fish oil supplemented with precursors and cofactors for membrane synthesis, viz. uridine-monophosphate (UMP), DHA and EPA, choline, folate, vitamins B6, B12, C, E, phospholipids, and selenium. **Plant sterol**: fish oil supplemented with phytosterols.

Behavior. Transgenic mice, fed with Control chow, showed poor spatial learning, hyperactivity in exploring a novel cage, and reduced preference to explore novel odours. In the Morris swim task, APP/PS1 mice had longer escape latencies than WT mice, indicative of impaired learning. The escape latency did not differ amongst APdE9 mice in the different diet groups when all days were considered. However, group differences emerged in the last two days of the task. In the post-hoc test, the Fortasyn group was superior to both Fish oil and Plant sterol groups. The groups did not differ in their swimming speed. Exploratory activity in terms of gross horizontal distance moved during two 10 min session in a new test cage differed significantly between the genotypes, such that APP/PS1 mice were hyperactive. No differences were observed among APP/PS1 mice on different diets, however. All fish oil containing diets increased exploration of a novel odour over a familiar one.

Amyloid pathology. None of the diets altered the levels of cortical insoluble (soluble only in 5M guanidine) Aβ1-40 or Aβ1/42. However, the Plant sterol diet significantly lowered the levels of PBS-soluble Aβ1-42 in the cortex. There was a similar but nonsignificant trend for the Aβ1-40 ($p > 0.10$). The amyloid load was around 4% in the hippocampus and 6% in the parietal cortex. In keeping with the reduced Aβ1-42 load as measured with ELISA, the Plant sterol group had the lowest amyloid load in the hippocampus. However, the difference between groups was not statistically significant ($F_{3,37} = 1.6$, $p = 0.12$). Nonetheless, One-way ANOVA revealed a significant group difference in the amyloid load in the parietal cortex. Tukey's post-hoc test showed that the Plant sterol group differed from the Fortasyn group. Similarly the relative surface area of CD45 immunoreactivity, representing activated microglia/macrophages was calculated and this revealed that the CD45 immunoreactive area was the smallest in the Plant sterol group.

Experiment 2 compared **Control:** as in Exp 1, **Fortasyn:** as in Exp 1, **Endothelial:** Fortasyn w/o UMP and phospholipids, and L-cystine supplementation instead of choline, **Mitochondrial:** Control + vitamins, calcium, biotin, D-ribose, L-aspartate and L-lysine, **Combination:** Fortasyn with added L-cystine. Mice began the special diets at 5 months and were sacrificed at 14 months after a month of behavioral testing. The test battery was complemented with two tests for fear and anxiety:
Marble burying task for object neophobia and elevated plus maze for fear of high and open places: the most widely used anxiety test in neuropharmacology.

Behaviour. Spatial learning and memory **-** The APP/PS1 genotype effect was confirmed and the impairment of TG mice was robust in all phases of the task. Spontaneous exploration - A robust APP/PS1 genotype related hyperactivity was confirmed. All experimental diets attenuated the hyperactivity, so that only APP/PS1 mice on Control diet differed significantly from the WT mice on Control diet in horizontal activity.

Conclusions for experiment 2. Further modifications of the Fortasyn diet did not provide any additional benefits in terms of behavioural outcome or brain amyloid pathology. None of the formulations caused overt adverse effects.

Overall conclusion. A fish-oil based formulation (Fortasyn) supplemented with precursors and cofactors for membrane synthesis significantly improved spatial learning and odor recognition of APP/PS1 mice. These data provide proof-of-principle evidence that long-term use of dietary supplements may affect the key pathological features of Alzheimer's disease.

WP7 - Cerebrovascular animal studies
The objective is **to evaluate the cerebrovascular effects of lipid-based nutrition***.* Impairment of the cerebral circulation in the brain leads to decreased energy metabolism causing white matter lesions and the production of β amyloid which may result in cognitive impairment and dementia. In this work package the transgenic APP/PS1 mouse and apoE4 and apoE ko mice as models for Alzheimer's disease, and impaired (cerebral) circulation during aging and AD were used. The *in vivo* influence of diets on brain circulation, white matter lesions, brain (energy) metabolism with MRI and MRS techniques, are assessed as well as cognition and cerebrovascular/Alzheimer pathology during aging.
Epidemiological studies have shown that vascular disorders, such as hypercholesterolemia, and atherosclerosis, are major risk factors for AD. Modifying these vascular-based risk factors via lifestyle, such as nutrition, can alter the risk of developing AD later in life. This study therefore evaluated the effect of diet components and combinations thereof, on circulation such as cerebral blood flow (CBF) and plasticity, cerebral blood volume and blood pressure in relation to brain structure, cognitive performance and synaptic density during aging in mice between 8 and 18 months with neuroimaging, cognitive testing and postmortem analyses. To address these issues, three distinct transgenic mouse models: the Alzheimer mouse model AβPPswe-PS1dE9, and models for vascular disease; the apoE knockout and the human apoEε4/ε4-carrier were used. It was shown that disturbances in cerebral circulation are present already at an early age in the AβPP-PS1 mouse model, and that these precede any substantial neuropathological alterations except for Aβ pathology which is due to the enormous promotor driven production of Aβ which runs independent from other physiological processes. Both apoE ko and the apoE4 model seem to be good models to study very early stages of neurodegeneration in aging as circulation and connectivity as well are already affected before apparent cognitive decline. Of all tested diets the multi-nutrient Fortasyn diet, containing n3 lc-PUFAs and UMP, choline, vitamins B6, B9, B12, C and E, folic acid, selenium and phospholipids was shown to be the most effective in improving cerebral blood flow and reactivity of the vessels, restoring neurogenesis and synaptogenesis and decreasing anxiety-related behavior and protecting neuronal and axonal integrity and connections between brain area. Therefore, it was concluded that changes in hemodynamics play a major role in the progression and aggravation of AD and that a multicomponent and multi-targeting diet simultaneously improving cerebrovascular health and enhancing neuroprotective mechanisms has the potential to inhibit the development of Alzheimer's disease in the early stage of the disease. The degenerative processes underlying the development and progression of Alzheimer's disease (AD) are complex and far from being completely understood. Rather than one single cause, AD is a multifactorial disorder, caused by a combination of different (risk) factors which affect the integrity

of the brain at multiple levels. Although ageing is the most important risk factor for AD, several other factors have been associated with an increased risk of developing AD. Epidemiological studies have shown that vascular disorders, such as hypercholesterolemia, and atherosclerosis, are major risk factors for AD. Modifying these vascular-based risk factors by means of changing lifestyle, such as physical exercise and nutrition, can thereby alter the risk of developing AD later in life. Dietary intake of typical Western diets, high in saturated fatty acids such as cholesterol, increases the incidence of cardiovascular disease and AD, whereas adherence to a Mediterrean diet, rich in omega-3 long-chain polyunsaturated fatty acids (n3 lc-PUFAs), vitamins and antioxidants, lowers the incidence of vascular disorders and reduces the risk of developing AD. This opens up new horizons in the search of preventative strategies for AD.

This WP demonstrated that it is possible to increase neurogenesis, vascular protection and learning, and reduce anxiety by diet. Moreover, it was shown that the combination diet gives excellent results in preventing the occurrence of the typical structural (DTI) damage present in AD model mice, relevance of sex differences and/or presence of genetic risk factors or other pathologies. E.g. atherosclerosis and apoE ε4 (apoE4) genotype are risk factors for Alzheimer's disease (AD) and cardiovascular disease (CVD). Sex differences exist in prevalence and manifestation of both diseases. These were investigated respective to aging, focusing on cognitive parameters in apoE4 and apoE knockout (ko) mouse models of AD and CVD. To our knowledge, no other studies investigating presynaptic density in aging female apoE4 or apoE ko mice are available. Sex-specific differences between apoE genotypes could account for some sex differences in AD and CVD.s
The effects of long-term consumption of a specific multi-nutrient diet in two mouse models for atherosclerosis and high cholesterol were tested. These data suggest that the specific dietary intervention has beneficial effects on early pathological consequences of hypercholesterolemia and vascular risk factors for AD.

In patients with Alzheimer's disease the severity of the loss of connections in the brain correlates with the severity of the disease. A new technique: diffusion-tensor magnetic resonance imaging to study the connections in the Alzheimer mouse brain which were decreased in AD patients was employed. This model can therefore be used to investigate future AD prevention strategies.

Proton magnetic resonance spectroscopy ((1)H MRS) is a valuable tool in Alzheimer's disease research, to investigate the metabolism in the brain. The metabolism of the brain tissue in adult and old Alzheimer mice was investigated and related it to their cognition and Alzheimer pathology. It was shown that a lower metabolism was related to impaired cognition and also increased Alzheimer pathology only during older age.

Lifestyle factors like diet may influence the onset and progression of Alzheimer's disease. Learning strategies and memory of 11-month-Alzheimer mice were analysed. The specific nutrient combination showed a tendency to improve searching behaviour in AD mice by increasing the use of a more efficient search strategy and improving their swimming efficiency by decreasing the latency to reach the former platform position.
Some diets, like the Mediterranean diet, are well known for their health-promoting effects. Many scientists have tried to pinpoint such effects of diets to single ingredients, but such attempts have not been very successful. Some combinations of ingredients were tested in an animal model of Alzheimer's disease showing that beneficial effects of diets may depend very much upon the specific combination of ingredients. The following results were achieved: combination diet normalizes blood pressure in aging, the same diet increased cerebral blood flow reflecting a better vasoactivity of the capillaries. A significant reduction of CBF, vasoactivity and white matter was found in 12 months APPswe/PS1dE9 mice compared to WT littermates and compared to WT littermates, APPswe/PS1dE9 mice showed more anxiety and hyperactivity and impaired learning, and increased inflammation and decreased neurogenesis.

WP8 - Clinical trial
The main objective of WP8 for the whole period of the project, is to investigate the effects of the nutrient combination Fortasyn in prodromal Alzheimer's disease through a double-blind placebo/control controlled 24-month study on cognition, biomarkers in plasma, CSF and atrophy rate as measured by MRI.

The LIPIDIDIET trial is one of the first randomized controlled trials (RCTs) with 2 years follow-up in patients with prodromal Alzheimer's disease aged 55 to 85 years old. Patients with prodromal AD have memory problems and evidence of Alzheimer brain changes (e.g. in cerebrospinal fluid or on brain scans), without dementia. Criteria for diagnosing prodromal AD are quite recent (Dubois et al, 2007) and so have not been used routinely in clinics. The trial investigates whether a medical food has protective effects on cognitive functioning, and also on the development of dementia, daily life functioning, depressive symptoms, and several biomarkers measured in blood, cerebrospinal fluid and brain scans. Safety and tolerance of the medical food are also investigated.
This LIPIDIDIET trial seeks to answer the question whether a medical food can maintain cognitive stability in patients with a high risk of developing AD dementia.
Response by the participating volunteers has been very positive, the study drink which contains the DHA based nutritional formulation (vanilla and strawberry) is well received and very few participants have thus far opted to leave the trial before finishing the first two year milestone (20%). Indeed, within the attached LIPIDIDIET extension study the first patients are already in their sixth year of intervention.

WP9 - Epidemiological studies
The objective of this WP is 2-fold: (1) to make dietary recommendations for brain health related to intake of dietary cholesterol, saturated and polyunsaturated fats, fish, and fruits and vegetables; and (2) to suggest guidelines for appropriate levels of blood lipids and adiposity measures for elderly people.

Specific tasks of this WP were to analyse the relationship between:
- blood cholesterol/lipid levels and AD and cerebrovascular disease in mid- and late-life
- adiposity factors and AD and cerebrovascular disease in mid- and late-life.
- dietary fat intake and habits in mid-life and AD and cognitive impairment
- fruit and vegetable intake in mid-life and AD and cognitive impairment
- the potential modifying role of apoE in relationship to AD and cerebrovascular diseases and the relationship to blood cholesterol levels and adiposity indices, and dietary intake, and AD and cerebrovascular disease.

Through fulfillment of the tasks listed above, the goals were to develop:
- a 'healthy brain diet' index in relation to AD and cognitive impairment which will also provide the corresponding dietary recommendations.
- a mid-life risk score for AD and cognitive impairment which is based on both diet and the blood levels of 'fat' factors.

Analyses of a number of study population show how strong the influence of diet is on the risk to develop dementia. More and more has been learnt about dietary components/dietary life-style factors are correlated with a reduced or increased risk for dementia. A recent study from our Consortium studied the changes in vascular factors three decades from midlife and late-life cortical thickness, vitamin E and D levels and forms and risk of cognitive impairment in older adults, homocysteine in AD and cerebrovascular pathology (post mortem), physical activity from mid- to late-life, BMI and AD risk. Not unexpected, this indicates that a healthy diet is beneficial, but more importantly, it enables identification what specifically should be avoided or not.

One such study investigated associations between plasma vitamin D and cognitive impairment, cerebrospinal fluid (CSF) biomarkers of Alzheimer's disease (AD), and brain tissue volumes. Results suggest that vitamin D may be associated with cognitive status, CSF Aβ42 levels, and brain tissue volumes (e.g. white matter, structures belonging to medial temporal lobe), which also relates to our findings in WP3.

Other factors such as overweight and obesity may increase risk for Alzheimer's Disease and other dementias occurring in later life or after age 65 years Body Mass Index (BMI) is a common measure of overweight and obesity and BMI is known to influence risk for dementia. Since BMI changes with age over the life course (increasing with increasing age during adult years, and decreasing with increasing age as one gets older) different dementia risk associations are observed in adulthood versus later life. In midlife (to age 60 years), higher BMI increases risk for dementia. In late-life, higher BMI is protective for dementia. In another study we explored these

measures in relation to 7-year mortality in long-lived Italian elderly, comprising a representative, age-stratified, population sample from Treviso, Italy, the Treviso Longeva (TRELONG) Study. Three hundred eleven men and 357 women, aged 70 years and older (mean age 84 ± 8 years) were followed for seven-year mortality, which was evaluated in association with BMI, Mini-Mental State Examination (MMSE) score, Activities of Daily Living (ADL), apoE genotype, and a variety of clinical and survey data. In separate age- and sex-adjusted analyses, BMI <18.5 kg/m(2), MMSE ≤24, and ADL <6, were associated with greater 7-year mortality among adults aged 70 years and older. In a multivariate model including all factors, MMSE ≤24, and ADL <6 were associated with greater mortality; BMI ≥30 kg/m(2) was protective. Here no interactions between BMI, MMSE, or ADL were found. When excluding those dying within 3 years of baseline, only an MMSE ≤24 was related to mortality. Higher MMSE score, higher ADL score, and higher BMI, independent of age, sex, and other factors, are markers for longer life among northern Italian adults aged 70 years or older. Global cognition, BMI, and physical functioning, assessed by short, simple tests are profound indicators of death within less than a decade.

When investigating the association of midlife and late-life body mass index (BMI) with late-life dementia/Alzheimer's disease and whether the association was independent of other obesity-related co-morbidities it was found that higher midlife BMI was related to higher risk of dementia and AD, independently of obesity-related risk factors and co-morbidities. Steeper decrease of BMI and low late-life BMI were associated with higher risk of dementia and AD. These findings highlight the importance of life-course perspective when assessing the association between BMI and cognition.

Vitamin E includes 8 natural antioxidant compounds (four tocopherols and four tocotrienols), but α-tocopherol has been the main focus of investigation in studies of cognitive impairment and Alzheimer's disease. We investigated all 8 vitamin E forms in 140 older individuals without cognitive impairment followed-up for 8 years. Elevated levels of tocopherol and tocotrienol forms were associated with reduced risk of cognitive impairment. The association was modulated by cholesterol levels. Various vitamin E forms might play a role in cognitive impairment, and their evaluation can provide a more accurate measure of vitamin E status in humans. We also found that that elevated homocysteine may contribute to increased Alzheimer-type pathology, particularly neurofibrillary tangles burden. This effect seems to be more pronounced in the presence of cerebrovascular pathology. It was found that:

A higher CAIDE dementia risk score (including hypercholesterolemia) was associated with more severe white matter lesions 20 years later, and with higher volume of white mater lesions and more severe medial temporal atrophy up to 30 years later.

Coronary heart disease (CHD) was associated with lower thickness in multiple brain regions, and lower total gray matter volume, particularly in people with longer disease duration (>10 years). Associations between CHD, cortical thickness and gray matter volume were strongest in people with CHD and hypertension in midlife, and those with CHD and declining blood pressure after midlife. No association was found between CHD and WML volumes. Based on these results, long-term CHD seems to have detrimental effects on brain gray matter tissue, and these effects are influenced by blood pressure levels and their changes over time. Cholesterol levels did not seem to influence these associations.

These data suggest that overnutrition, defined as excess energy intake, manifested as overweight and obesity, is not healthy for the brain, especially in mid-life. It is important to maintain a healthy body weight within the healthy range of BMI (BMI, 18.5-24.9 kg/m2) for optimal brain health.

Our findings from the CAIDE study indicated that moderate coffee consumption at midlife was associated with a decreased risk of dementia and AD in late-life. Tea consumption, however, showed no association with dementia or AD in this population.

Combined dietary patterns - People who ate the healthiest had an 86–90% decreased risk of dementia and a 90–92% decreased risk of AD compared with persons whose diet was considered least healthy. Adjustments for various confounders did not change the results. When the data were reanalysed for dementia also including diagnoses from the patient records for the non-participants in the follow-up, people in the high-adherence group showed a tendency towards a decreased risk of dementia compared with the group with a low adherence to a healthy diet.

In summary, these and other results from the LIPIDIDIET project emphasize that unhealthy dietary factors at midlife may increase the risk of developing dementia/AD later in life and have a negative effect on several cognitive domains, whereas healthier dietary choices may act in the reverse manner. The current data suggests that healthy dietary choices at midlife may increase the

possibility for brain health later in life. Dementia is a complex condition and multiple factors influence the risk during the life course. Diet is only one piece of the puzzle. A healthy diet should be part of a generally healthy lifestyle including also physical activity, cognitive and social activities, and regular medical check-ups (particularly for persons who have chronic cardiovascular, diabetes, and other conditions). The fact that there are several modifiable risk factors for dementia creates a critical window of opportunity for risk reduction.

A guide to a healthy diet is published on the LIPIDIDIET website.

4 Description of the potential impact and the main dissemination activities and exploitation of results

LIPIDIDIET has **advanced the state of the art in the field of nutrition and in research into the ageing process** by exploring the contribution of dietary lipids and other nutritional molecules on the development of AD and vascular dementia. The LIPIDIDIET project is unique, as it combined molecular and preclinical approaches with the most recent epidemiological data about the impact of dietary habits, genetic predispositions and measurable biomarkers on lipid metabolism with cellular and animal disease models to get a thorough understanding of the underlying mechanisms. This newly acquired information about the relationships between molecular pathways, the intake of certain dietary lipids and brain vascular and amyloid changes forms the basis for the rational design of dietary interventions. These interventions were tested and validated in preclinical cellular and animal models and in clinical trials with patients in a disease prevention rather than treatment design. This significant effort was made possible in LIPIDIDIET by a **large, collaborative European network of experts** in brain lipid metabolism, cellular and animal models of neurodegenerative diseases, epidemiology, clinical neurology, nutrition and manufacturing of specialized nutritional products.
Dementia in the rapidly ageing population is becoming such a massive challenge to the European health care system, particularly in light of a persisting situation in which pharmaceutical drugs offer no cure. It is well established that delaying disease onset by even a short period of time can considerably reduce the total number of dementia patients; this would consequently lessen the related need for socio-economic and societal resources for care. Existing drugs provide symptomatic relief for a limited time for a minority of the patients treated only. The patient population studied in LIPIDIDIET is still at an early stage of the disease process. They still suffer from a very mild cognitive impairment only. Most of them will not progress from dementia for several years to come. Drug treatment possibilities, even in clinical trials, are severely hampered by the critical issue of potentially exposing patients to drug-induced side-effects.
Unfortunately, new effective drug therapies for dementia are not expected to become available soon. This is especially regrettable for those who stand to gain most from an effective intervention: patients who have not yet developed the symptoms of dementia. In these patients, the disease is still dormant, but bound to surface within a few years (**prodromal AD**). Identifying these patients has only recently become technically feasible with sufficient diagnostic sensitivity and specificity.

In contrast, nutrition-based treatment is well-tolerated and is an excellent choice for prodromal AD. Our study gives clear evidence that **dietary intervention is currently the preferred choice**. This is critical, because patients are required to adhere to a chosen treatment for many years. Indeed, we observed that the patients in the LIPIDIDIET clinical trial followed the intervention scheme very strictly. Additionally, even in the long-term follow-up studies (up to a continuous treatment of 6 years) the drop-out rate (patients who stopped participating in the clinical trial) was very low. The preference for dietary intervention is confirmed in the recent FINGER study, which tested interventions with diet, physical and cognitive activity and other activities in those at risk of dementia; in this study adherence was best for the dietary intervention.

This is remarkable, because it was assumed that changing dietary habits and maintaining them would be very difficult to achieve. LIPIDIDIET and FINGER data now clearly indicate that **dietary changes are readily achievable and sustainable for an elderly population that is aware of their increased personal risk for cognitive decline**. Moreover, targeted dietary changes are **accessible to everyone, which expands the potential impact** of these findings far beyond the clinical situation.

To further increase the accessibility to LIPIDIDIET results, the LIPIDIDIET consortium **provides dietary recommendations for healthy ageing and the well-being of the elderly based on scientific evidence**. The **identification of both beneficial and harmful dietary ingredients** is a necessary step towards formulating of dietary recommendations. By providing solid scientific data to support health claim relevant findings, the LIPIDIDIET project is expected to make a considerable contributribution to the introduction of nutritional compositions to help people age well, while retaining a high quality of life.

In order to determine dietary recommendations for the elderly, it is important to have **knowledge of** historical and current lifestyle factors and environmental exposures. Then, to develop protective and therapeutic interventions, scientists must simultaneously view pathological processes on a microscopic scale, as well as a view of people as a whole. The basic, clinical, and epidemiologic components of this process provide the knowledge needed to develop dietary recommendations for healthy aging. To this end, we provided and continue to provide data to assess age-related disease risk and to **better understand how health can be managed through specifically designed, scientifically-based nutrition**. Thus, the LIPIDIDIET research design can be used as a model not only for research into dietary interventions, but also as a way for health care professionals and consumers/ patients to fully understand the benefits and limitations of a nutritional approach. In this respect, LIPIDIDIET adds value through hundreds of LIPIDIDIET, LIPIDIET and project-related publicly available scientific publications which have been validated by external experts and provide an extensive view of the project results.
The information gathered during the project also **provides sound scientific data** that the food industry and safety boards could use **to substantiate health and nutrition claims**. Nutritional research also contributes to better **food quality and safety** and increases **Europe's competitiveness**. **Competitiveness is an ongoing quality that relies** on constantly pushing boundaries to move forward.

To link experimental data with human dietary lifestyle, the project utilises epidemiological information. Therefore, large, well-described population-based studies with different baseline ages, long follow-up times (20-35 years), and measurements of numerous risk factors and health-related outcomes at several time points (midlife and late-life) were used for epidemiological investigations. These databases can be interlinked and used in parallel, which has led to the creation of new databases with large numbers of subjects and will continue to do so. This makes it possible to compare risk factor profiles in different age groups, genders and the effects of different follow-up times.

By increasing the numbers of individuals for whom we can identify innate susceptibility factors for dementia and cerebrovascular diseases, we can gain a **better understanding of Nutrigenomic and gene-environment interactions** and associations. This paves the road to develop **personalized guidelines** for brain health based on genetic and lifestyle factors. For example, individuals with the apoE ε4 allele are a susceptible group which could be targeted to reduce their disease risk through dietary interventions.
We expect that the development of dietary anti-dementia guidelines, like the cholesterol story in cardiovascular disease, will help educate people about the importance of healthy eating habits and life styles.

As a whole, our efforts in the LIPIDIDIET project have led to **comprehensive dietary guidance** which addresses specific issues including dietary recommendations, novel foods, and modified foods. Where necessary, the guidance includes specially targeted nutrition to help patients in nursing homes, clinics and at home. Progress within this project, as well as generally in this area of research, has strongly increased the opportunities for diet-based or diet-supported preventive approaches for cognitive performance in aging, Alzheimer's disease and vascular dementia.

The approaches used by LIPIDIDIET resulted:

- in practical advice,
- knowledge and products which do not impose any foreseeable or long-term adverse risks,
- are sustainable,
- ready and easy to implement,

- provide choices and alternatives for the individual, and as such are better suited to effectively address the European population, including parts of the population who are typically critical towards preventive approaches,
- will help lower socio-economic costs and strengthen the competiveness of the European scientific and commercial sector, and
- last, but not least, the approaches help to improve the quality of life within the healthy aging concept.

The LIPIDIDIET project has already become part of other projects which will increase the overall impact of LIPIDIDIET. An example is **MindAD** (JPI/JPND funded), in which all major completed interventional long-term dementia prevention projects are combined, to further increase the analytical power and to create the most synergy in dementia prevention efforts. Moreover, **to maximize the transfer of results from the project to the general public**, implementation into the European health care system is actively being advanced. The Program Prévention-Démence – Luxembourg (**PDP**), conducted by LIPIDIDIET participants, is one such project. It focuses on **nation-wide implementation of dementia prevention for preclinical and prodromal AD patients**. Performed in one of the smaller member states of the EU, it provides an effective means to model secondary dementia prevention activities for other regions and larger states.
Scientists from LIPIDIDIET are frequently invited to present their results and to provide **advice to national and international governmental and policy** related meetings on dementia prevention and healthy aging. This illustrates that our research, while very molecular, complex and multifaceted, is designed to be readily implemented. It provides essential information on topics ranging from dietary strategies, genetic risk groups, and early diagnosis to specialized medical food. Importantly, it offers concrete advice that is relevant for both the general public and policy makers.
Our results, impact and directions for future development ensure that the LIPIDIDIET project continues to contribute towards the scientific, medical and societal goals beyond its funding period. Highly ambitious and visionary research requires substantial resources and bears the risk of not achieving its original goals. However, as our results give clear evidence for, research is indeed an **effective investment to address and advance health care, (socio-) economic and societal issues and the needs of individuals** and of **modern highly knowledge-driven societies,** like that of the European Union.

THE LIPIDIDIET CONSORTIUM: