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# A 2-year Double-Blind RCT Follow-up Study with Fermented Papaya Preparation (FPP) Modulating Key Markers in Middle-Age Subjects with Clustered Neurodegenerative Disease-Risk Factors

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## Abstract

In recent years a number of studies have reported the significant relationship between metabolic syndrome and neurodegenerative disease. There is accumulating evidence that the interplay of combined genetic and environmental risk factors (from diet to life style to pollutants) to intrinsic age-related oxi-inflammatory changes may be advocated for to explain the pandemic of neurodegenerative diseases. In recent years a specific Fermented Papaya Preparation (FPP) has been shown to significantly affect a number of redox signalling abnormalities in a variety of chronic diseases and as well in aging mechanisms either on experimental and on clinical ground. The aim of the present study was to evaluate FPP use in impending metabolic disease patients with potentially neurodegenerative disease clustered risk factors. The study population consisted of 90 patients aged 45-65 years old, with impending metabolic syndrome and previously selected as to be ApoE4 genotype negative. By applying a RCT, double-blind method, one group received FPP 4.5 g twice a day (the most common dosage utilized in prior clinical studies) while the other received an oral antioxidant cocktail (trans-resveratrol, selenium, vitamin E, vitamin C). Then, after 21 month treatment period, a selected heavy metal chelator was added at the dosage of 3 g/nocte for the final 3 months study treatment. The parameters tested were: routine tests oxidized LDL-cholesterol, anti-oxidised LDL, Cyclophilin-A (CyPA), plasminogen activator inhibitor-1 and CyPA gene expression. From this study it would appear that FPP, unlike the control antioxidant, significantly decreased oxidized-LDL and near normalizing the anti-Ox-LDL/Ox-LDL ratio (p<0.001) although unaffecting the lipid profile *per sè*. Moreover, only FPP decreased cyclophilin-A plasma level and plasminogen activator-inhibitor (p<0.01) together with downregulating cyclophilin-A gene expression (p<0.01). Insulin resistance was only mildly improved. Heavy metals gut clearance proved to be effectively enhanced by the chelator (p<0.01) and this was not affected by any of the nutraceuticals, nor it added any further benefit to the biological action of FPP.

**Keywords:** Fermented Papaya Preparation (FPP); Redox balance; Antioxidant; Neurodegenerative disease; Caviarlieri

## Introduction

Starting in the early 90' gold standard electron spin resonance studies had shown that a functional food consisting of fermented papaya (FPP, ORI, Oxidative Stress laboratory, Gifu, Japan) exhibited a powerful anti-oxidative activity on *in vitro* cerebral cells [1] as well on *in vivo* epilepsy experimental model, where the epileptogenic monoamine neural release was consistently reduced [2]. Later on, a Japanese group proved the capacity of FPP to reduce the derangement of oxidant/antioxidant balance at the brain level in elderly rats and in experimental ischemia-reperfusion induced cerebral damage as well [3,4]. In recent years our group has shown that FPP could beneficially effect on clinical ground a number of redox signaling abnormalities in

a variety of chronic diseases [5,6] as well as some key aging mechanisms [7,8].

Recent studies using transgenic mice have shown the importance of proinflammatory Cyclophilin A (CypA)-metalloproteinase-9 in blood-brain-barrier integrity [9] while other have proved that a specific inhibitor of CyPA could reduce neuroinflammation, improve motor neurons activity and prolong survival in a mouse model of amyotrophic lateral sclerosis [10]. The potential neuroprotective effects of FPP are at the moment the target of a clinical study on Parkinson's disease patients by the neurology group of Nordera. This group has reported some preliminary promising results such as a reduction of motor scores of the Unified Parkinson Disease Rating Scale, improvement of Activity of Daily Living performance and of redox biochemistry (G. Nordera, personal communication at Oxidative Stress in Health and Prevention, September 24, 2014 http://www.centrostressossidativo.it/video-congresso-stress-ossidativo/). This latter aspect has received further support by the fine biochemical

analysis of same patients cohort by Bolner et al. [11]. Moreover, it has been recently shown that FPP could dramatically decrease the oxidative stress parameters in established Alzheimer Disease (AD) patients [12].

In recent years a number of studies have reported the significant relationship between metabolic syndrome and neurodegenerative disease [13-15]. Indeed, current views on dietary-related diseases and also neuroimaging data have reported the association between dis-metabolic patterns and neurodegenerative damage [16] where low-grade inflammation and insulin-resistance seem to be among main background factors involved [17-19]. As for metabolic syndrome, different groupings of the following metabolic parameters have been listed in each classification, such as insulin resistance, hypertriglyceridemia, low HDL-C, obesity/increased waist circumference, altered glucose tolerance/diabetes mellitus, hyperinsulinemia, microalbuminuria and hypertension. In the current study we referred to the definition of MS as outlined by the American Heart Association and the National Heart, Lung, and Blood Institute [20].

A further open issue regarding the multifactorial pathophysiology of neurodegenerative disease is the potentially detrimental role played by occupational and environmental exposure to heavy metals [21-25]. Indeed, while some metals are involved in physiological enzymatic reactions, once they accumulate in the brain, especially if overtly acting as xenobiotic, may give raise to oxidative stress phenomena with mitochondrial dysfunction, protein misfolding and aberrant autophagic processes [26-28]. As a matter of fact the update literature suggests that the aggregation of disease-related proteins during physiological aging can be advocated for by abnormal protein homeostasis observed. Such abnormalities may thus represent a biomarker of aging that could modulate life span and cause neurodegeneration. Albeit not fully unfolded as yet, it seems that the interplay between combined genetic and environmental risk factors (from diet to life style to pollutants) with intrinsic age-related oxi-inflammatory changes may be advocated for to explain the current increasing surge of neurodegenerative diseases. Given all above, the current challenging quest of medical community is mostly focused to track down early biomarkers of likely modifiable causative factors together with possible beneficial approaches within a multifunctional therapeutic armory. The aim of the present study was to evaluate FPP use in impending metabolic disease patients with potentially higher risk to develop a neurodegenerative disease.

## Methods

### Ethics

All procedures were approved by an independent Ethical Committee for nonpharmacological research (ReGenera Research Group for Aging Intervention, protocol FPP-MSNEU-10/2015). Each subject recruited for the study was fully informed and treated in compliance with the guidelines of the Declaration of Helsinki.

### FPP

The FPP used in the present study was obtained from *Carica papaya* L. cultivated in Hawaii, following yeast fermentation for 10 months and pharmaceutical-grade batch-to-batch control at the Osato Research Institute (Gifu, Japan). The final composition of FPP per 100 g is as follows: 90.7 g carbohydrates, 17 µg vitamin B6, 2 µg folic acid,

2.5 mg calcium, 16.9 mg potassium, 240 µg niacin, 4.6 mg magnesium, 14 µg copper, 75 µg zinc, 16 mg arginine, 6 mg lysine, 5 mg histidine, 11 mg phenylalanine, 9 mg tyrosine, 18 mg leucine, 9 mg isoleucine, 5 mg methionine, 13 mg valine, 11 mg glycine, 8 mg proline, 37 mg glutamic acid, 11 mg serine, 8 mg threonine, 27 mg aspartic acid, and 2 mg tryptophan. This formulation was chosen also to replicate a similar mixture reported on the market to have antioxidant effect in humans. Other claimed "fermented papaya" extracts available on the Italian market were excluded since prior in-house electron spin resonance testing had ascertained their poor to nil antioxidant effect (Mantello P. ORI Oxidative Stress Research Lab, Gifu, Japan. In-house data).

### Study design

The study population consisted 90 patients ranging from 45 to 65 years old, with impending metabolic syndrome (2 parameters out of the above-mentioned classification) and 30 age-matched healthy volunteers (Table 1). Patients with secondary hypertension, cardiomyopathy, severe abnormalities of liver, thyroid and kidney function, cerebrovascular diseases, grossly elevated total cholesterol (>280 mg/dl or LDL >180 mg/dl), malignancies and history of coronary bypass surgery or on insulin treatment were excluded from this study. Exclusion criteria were also the consumption of antioxidant or other supplements in the past 3 months.

All patients were screened for ApoE4 polymorphism to rule out the presence of a known risk gene pattern for neurodegenerative disease. This was a RCT, double-blind study, with FPP 4.5 g given twice a day vs. common antioxidant cocktail (trans-resveratrol 20 mg, selenium 60 mcg, vit E 30 mg, vit C 100 mg, papaya flavour) twice a day. Then, after 21 month continuous treatment period, an heavy metal chelator (talc-size, higher potency chabasite-phillipsite zeolites naturally-occurring mixture, Novagenics-SOL Ltd., Hong Kong) was added at the dosage of 3 g/nocte for further 3 months to complete the 24 months study (Figure 1).

At entry 1, 3, 6, 12, 21 and 24 months, biological samples were collected (BD Vacutainer sampling tubes) to assess biochemical assessments as below mentioned. Blood serum was isolated by centrifugation and stored at under −70°C until analysis. Repeated thawing and freezing were avoided.

### Parameters

**Pre-recruiting gene testing:** Ten millilitres of peripheral whole blood was withdrawn from an antecubital vein of all recruited subjects. Genomic DNA was extracted by a modified salting-out method (by precipitating DNA with the use of 96% ethanol). This was processed to ApoE genotyping with modifications in the multiplex amplification refractory mutation system PCR (Multi-ARMS PCR). The primer sequences used in the PCR analysis were as follows: APOE rs7412 F:ATGCCGATGACCTGCAGA R:ACTGGCGCTGCATGTCTT Product size (bp) 684; rs429358 F:TCGGAACTGGAGGAACAACT R:TACACTGCCAGGCGCTTCT Product size (bp) 260. Genomic DNA (50 ng) amplification (ABI Prism 310, Perkin Elmer, USA) comprised 30 cycles (94°C for 5 min, 94°C for 30 s, 65°C for 30 s, and 72° for 30 s), followed by a cycle of 10 min at 72°C for the final chain extension. Moreover, for the analysis of restriction fragment length polymorphism, the amplification product was digested with HhaI, followed by 5.0% agarose gel electrophoresis for 2 h and 30 min, stained with electro-fluorescent red stain (ThermoFisher, USA) and

UV light-visualization of the DNA fragments. As stated above, ApoE4 subjects were excluded from the study.

| Parameters | Healthy Control | FPP-treated | Antioxidant-treated |
|---|---|---|---|
| Total no. (male/female) | 30 (15/15) | 45 (33/18) | 45 (28/17) |
| Mean age | 67 | 71 | 66 |
| Mean BMI (range) | 23 (21-25) | 27 (23-35) | 26 (24-34) |
| Family history of diabetes | 1/30 | 14/45 | 21/45 |
| Family history of hypertension | 1/30 | 17/45 | 18/45 |
| Overt diabetes | NA | 6/45 | 11/45 |
| Duration of diabetes (years) | NA | 4 | 3 |
| Dyslipidemia | 0/30 | 28/45 | 31/45 |
| Smoking | 4/30 | 7/45 | 12/45 |
| Waist (cm) | 82 | 98 | 99 |
| Waist/hip ratio | 0.82 | 0.92 | 0.95 |
| Mean Systolic Blood Pressure (mmHg) | 122 | 159 | 161 |
| Mean Diastolic Blood Pressure (mmHg) | 65 | 78 | 81 |
| Physical activity | 15/30 | 6/45 | 12/45 |

**Table 1:** Anthropometric, clinical and biological parameters in studied subjects.
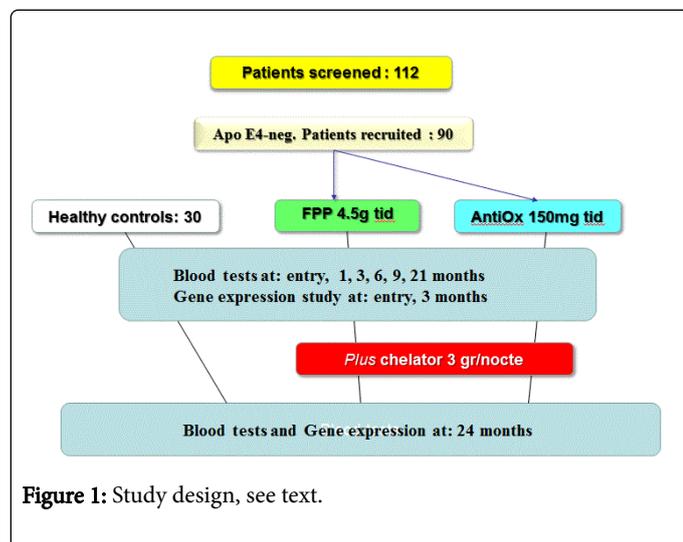


**Figure 1:** Study design, see text.

**Biochemical tests:** routine tests (WBC, transaminases, urea, creatinine, glucose, glycated haemoglobin, uric acid, HOMA test, LDL and HDL cholesterol) were assayed by using the 7060 Automatic Biochemical Analyzer (Hitachi, Ltd, Tokyo, Japan). Oxidized cholesterol, anti-oxidised LDL, ratio of anti-ox-LDL/ox-LDL cyclophilin-A, plasminogen activator inhibitor-1 were tested as below.

**Oxidised LDL and anti-oxidised LDL antibodies assessment:** These parameters were obtained by fractionation of LDL and preparation of oxLDL as antigen in the antibody detection assay, according to what reported by Ketelhuth et al. [29]. For this purpose a commercially available ELISA kits (ChusaBiotech Co. Ltd., China), was employed. Briefly, microtiter plates were coated with 10 g/ml in PBS of either native LDL or MDA-LDL to measure anti-oxidized LDL antibodies. Serum samples were diluted 1:100 in 1% BSA/PBS and incubated for 2 h at 37°C and overnight at 4 C. Afterwards, the plates were washed four times with alkaline phosphatase-conjugated anti-human IgG (Sigma Chemical, St. Louis, MO) and left for 3 h at room temperature. The reaction was halted after 60 min with 1% BSA/PBS for 2 h at room temperature. The absorbance was at 450 nm using a Multiskan RC ELISA reader (Thermo Fisher Scientific, USA). The binding of antibodies to oxidized LDL was determined by subtracting native LDL binding value from the one of MDA-LDL while the results were expressed as an Optical Density (OD).

**Measurement of cyclophilin A in plasma:** Cyclophilin A concentration in plasma was assayed with quantitative sandwich enzyme immunoassay kit (Wouhan-UCSN BioScience, Wouhan, China). Briefly, a monoclonal antibody specific for CypA was pre-coated onto a microplate. Standards and samples were put into the wells and CypA present is bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for CypA is added to the wells. Plates were washed three times with PBS and a substrate solution was added to the wells. The following dose-dependent color appearance was then halted by an acid solution and the color intensity measured by a reader as above. There was no evidence of any detectable cross-reactivity with any other tested proteins. All samples were analyzed in triplicate and samples with a CV >10% were discarded.

**Determination of plasminogen activator inhibitor-1:** PAI-1 serum concentration was measured by sandwich enzyme ELISA (PAI-1; DSE100; R&D Systems). Active PAI-1 levels were calculated from a standard curve constructed by using recombinant human PAI-1. By this method either active or latent forms of PAI-1 are detected [30]. Intra and inter-assay coefficients of variation of PAI-1 antigen were 3.9% and 4.8%, respectively.

**Cyclophilin-A gene expression study:** Reverse transcriptionpolymerase chain reaction (RTPCR) was used to determine their expression at 0, 90 days and at the end of the 24 month study period. Briefly, cells were lysed by Ambion lysis buffer (Ambion, Carlsbad, CA, USA) for 20 min and the lysates were mixed with same volume of 64% ethanol. The lysate products were put into a column and centrifuged at $10,000 \times g$ for 1 min. Afterwards, a buffer consisting of 700 μl buffer 1 and 500 μl buffer 2/3 (Genosolution, Ltd., Shenyang, China) was used to wash the column. After incubation with 50 μl buffer (GenePharma Co., Ltd.), the resulting flow was collected and 50 μl elution buffer was added and a further centrifugation was performed. Next, 1 μl DNAse I was added to 20 μl of RNA solution with suitable DNAse I buffer (GenePharma Co., Ltd.) and incubated at 37°C for 2 h. The DNAse I was removed using DNAse reagent and purified RNA obtained by a centrifugation at $10,000 \times g$ for 1 min. The quantity of total RNA was measured by optical density at 260 nm and the procedure checked through 1.5% agarose gel electrophoresis. RT was run in a 20ml solution containing 3 μg total RNA using the Revert cDNA Synthesis kit (Toyobo Biotech, Co., Ltd., Shanghai China). PCR was done in a thermal cycler after prior denaturation at 94°C for 5 min, followed by amplification for 30 cycles of denaturation at 94°C for 40 s, annealing phase at 65°C for 1 min followed by 72°C for 1 min. Afterwards, 5 μl PCR material was isolated by electrophoresis on a

1.5% agarose gel and stained by ethidium bromide. The following primer was used to carry out the test: CypA forward, 5'GTC AAC CCC ACC GTG TTC TTC3', and reverse, 5'TTT CTG CTG TCT TTG GGA CCTTG3' and glyceraldehyde3phosphate dehydrogenase (GAPDH) forward, 5'ACC ACA GTC CAT GCC ATCAC3', and reverse, 5'TCC ACC ACC CTG TTG CTGTA3'. The PCR products were subjected to electrophoresis on a 1.4% agarose gel and the results were quantitatively assessed by Gelmatrix Scan software (BioMatrixscan Inc., Shanghai, China).

### Faecal heavy metal measurement

A 15 mg of stool sample was collected and an inductively coupled argon plasma mass spectrometry (ICP-MS) with an Agilent 7500ce device was used to test the following heavy metals: lead, arsenic, mercury, cadmium, nickel and beryllium. According to the IUPAC guideline [31] the reference value was defined within the 95% confidence interval of the 95th population percentile. The results in the specimens were expressed as µg/L. Data were expressed on a dry weight basis so to avoid the variability related to watery component of the sample.

## Results

### Routine tests

Blood tests (WBC, creatinine, urea, glucose, glycated haemoglobin, uric acid, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, transaminases, gamma-GT, alkaline phosphatase, bilirubin) were not affected by any of the nutraceuticals employed, except a not significant trend improvement of HOMA test in the FPP-supplemented group (data not shown).

### Oxidized LDL cholesterol and anti-oxidised LDL antibodies

Whereas the group treated with antioxidant cocktail did not show any statistically significant difference in the oxidised lipid asset, patients treated with FPP showed a significant improvement of these parameters (Figure 2). In particular, the oxidized LDL cholesterol was normalised by this intervention (p<0.001 vs. baseline). This variation was significantly detectable starting from the third month observation period while the anti-Ox-LDL reverted to near to normal level from 12 months observation time onwards (p<0.05, Table 2). There was no significant correlation between these parameter and main clinical history data (presence or duration of diabetes, hypertension or dyslipidemia). No significant gender difference was detected as for these parameters are concerned.

### Serum level of cyclophilin-A and plasminogen activator inhibitor-1

At baseline there appeared a significant correlation between plasminogen activator inhibitor-1 and uric acid (r: 0.62, p<0.05, not shown). As compared to baseline and to antioxidant cocktail control, FPP showed to significantly decrease serum levels of cyclophillin-A from 1st month observation (p<0.01, Figure 3) and plasminogen activator inhibitor-1 from 6 month observation (p<0.01, Figure 4). A significant correlation appeared between cyclophillin-A and oxidised-LDL (r: 0.69, p<0.05, not shown) but not with plasminogen activator inhibitor-1.

### Cyclophilin-A gene expression study

At baseline observation, CyP-A gene expression was comparable with what observed in healthy controls (Figure 5). However, as compared to antioxidant cocktail-treated group, FPP enabled a significant upregulation at 3 and at 24 months (p<0.05, Figure 5).

### Faecal heavy metal assessment

There was no correlation between this parameter, either when analysed as a single heavy metal or as a whole pooled value and any of the biochemical or gene expression values measured.

Irrespective of the group, all patients showed an overall abnormal faecal metals profile with the exception of nickel and arsenicum.

Unlike FPP or antioxidant control, the chelator-treated group showed a significantly greater faecal elimination of heavy metals (namely: mercury, cadmium, lead and beryllium (Table 3, p<0.05 vs. baseline and antioxidant combination). There was a not significant trend correlation between cumulative heavy metal values (either at baseline or after treatment) with oxidised-LDL or its antibody (data not shown).

## Discussion

Oxidative modification of LDLs may be a prerequisite for the rapid accumulation of LDLs within macrophages to form foam cells; indeed, oxidized LDL has been found in extracts from atherosclerotic lesions [3]. Oxidative modification of LDLs induces also the transformation of immunogenic epitopes in the LDL molecule, which lead to the formation of antibodies against oxidized LDLs in the serum [4]. Nevertheless, the clinical importance of these autoantibodies is still under discussion. For example, in patients with diabetes, no association has been found between anti-oxidized-LDL antibodies and microvascular complications [11], nor has an association been found with the levels of cholesterol in patients with heterozygous hypercholesterolemia [32] or with the intensity of serum oxidizability [33]. Others have reported an inverse relation between levels of cholesterol and anti-oxidized-LDL antibodies in the general population [34] and this seems to agree with the finding of an inverse link between IgM autoantibodies to oxidized LDL and carotid artery atherosclerosis [35]. The preliminary data of our study confirms that FPP unlike the control antioxidant, specifically decreased oxidized-LDL and, on the long run, also enhanced its antibodies concentration, although not modifying the lipid profile. Unlike what reported by Balada et al. [36], we did not notice any significant gender difference as for the levels of anti-oxidized LDL antibodies. While brain cholesterol metabolism is separated from the systemic circulation, oxidised cholesterol moieties may damage the functional integrity of blood-brain barrier with potentially detrimental effects on neuronal population [37]. The same group has also shown on an experimental model that oxidised-LDL lipids and 27-OH-cholesterol may trigger Aβ production by GSH depletion and β-secretase-1 activation in neuronal cells [38]. It is noteworthy mentioning that a recent experimental study connects a fat-based/high calorie diet with metabolic syndrome, oxidative stress and significant hippocampal and temporal area degeneration together with memory dysfunction [39]. This evident histopathological finding is also in agreement with one of the current clinical hypothesis [40].
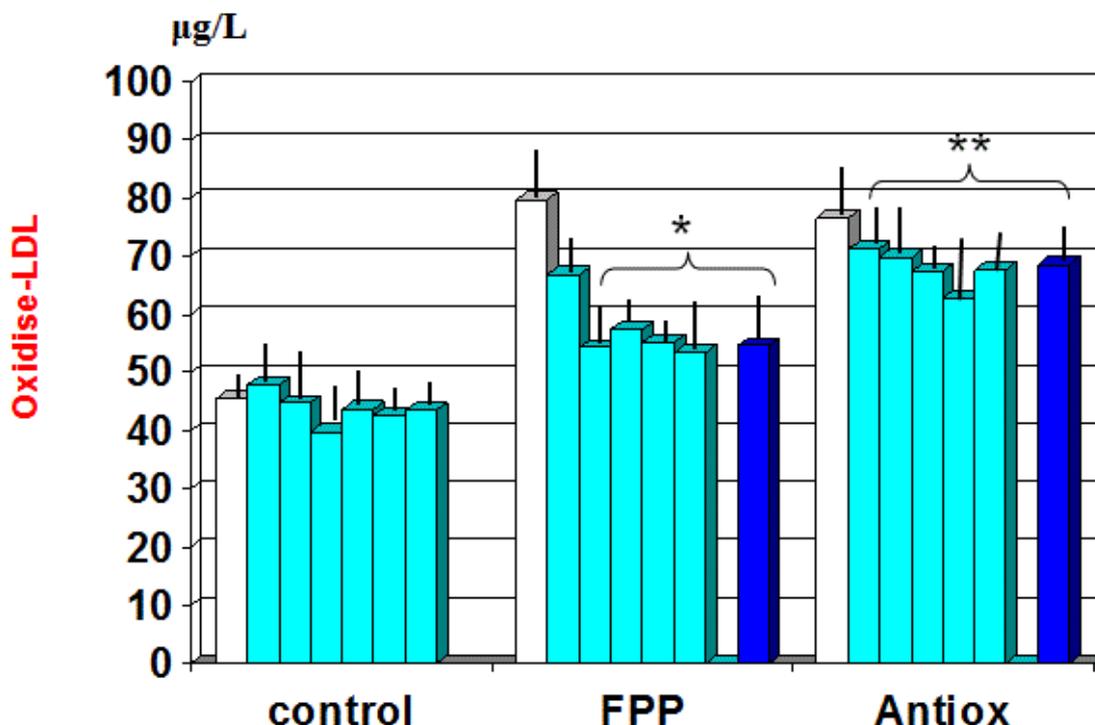
**Figure 2:** Variation of plasma level of oxidised-LDL during treatment with the two nutraceuticals formulas. *p<0.001 vs. baseline values; **p<0.01 vs. FPP-treated group.

| Anti-ox-LDL EU/mL (18.2 ± 3.4) | Entry | 6 months | 12 months | 21 months | 24 months |
|---|---|---|---|---|---|
| FPP | 28.1 ± 10.2 | 28.2 ± 9.3 | 21.4 ± 14.5[*] | 21.2 ± 10.7[*] | 20.5 ± 7.9[*] |
| AntiOx mixture | 26.8 ± 12.9 | 27.1 ± 8.8 | 27.4 ± 6.4 | 26.9 ± 13.3 | 25,7 ± 16.1 |

**Table 2:** Comparison of study parameters between the two treatment groups. In brackets: Values in healthy control subjects. *p<0.05 vs. baseline and vs. Antioxidant cocktail.

Moreover, only FPP decreased cyclophilin-A plasma level and plasminogen activator-inhibitor (PAI-1) (p<0.01). During inflammatory processes and following oxidative stress, CyPA is released into the extracellular compartment by inflamed cells [41,42] as a tentative protection response but this factor ends up being a potent chemoattractant for human monocytes and neutrophils. Cyclophilin A has indeed been shown to be more strongly associated with oxidative stress and inflammation than C-reactive protein [43]. Interestingly, prior small open pilot in-house studies testing same cohort of patients with an omega-3-rich sturgeon-based supplement (Caviarlieri, Swiss cap) did not beneficially affect the lipid profile, oxidised lipid parameters or CyPA. On the contrary, this latter variable showed a worsening change either as plasma level or as gene expression, making it a not advisable choice in metabolic syndrome or diabetes (J. Cervi, manuscript in preparation). The brain is characterized by a low content of antioxidant systems and the damage caused by oxidative stress is one of the earliest pathophysiological events in the development of Alzheimer's disease where plasma oxidized-LDL level has been positively correlated with disease severity [44].

A further independent cardiovascular risk factor [45] as well co-aggravating parameter in metabolic syndrome [46] is represented by PAI-1, the major regulator of fibrinolysis and a stress-related factor. As mentioned above, unlike the antioxidant cocktail and the recent negative study testing high dose tocotrienol [47], FPP supplementation brought about a significant normalization of altered values of this parameter.
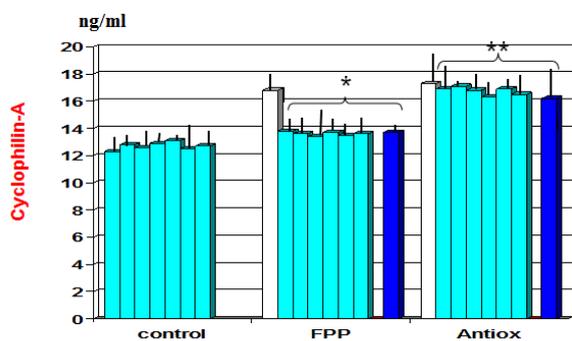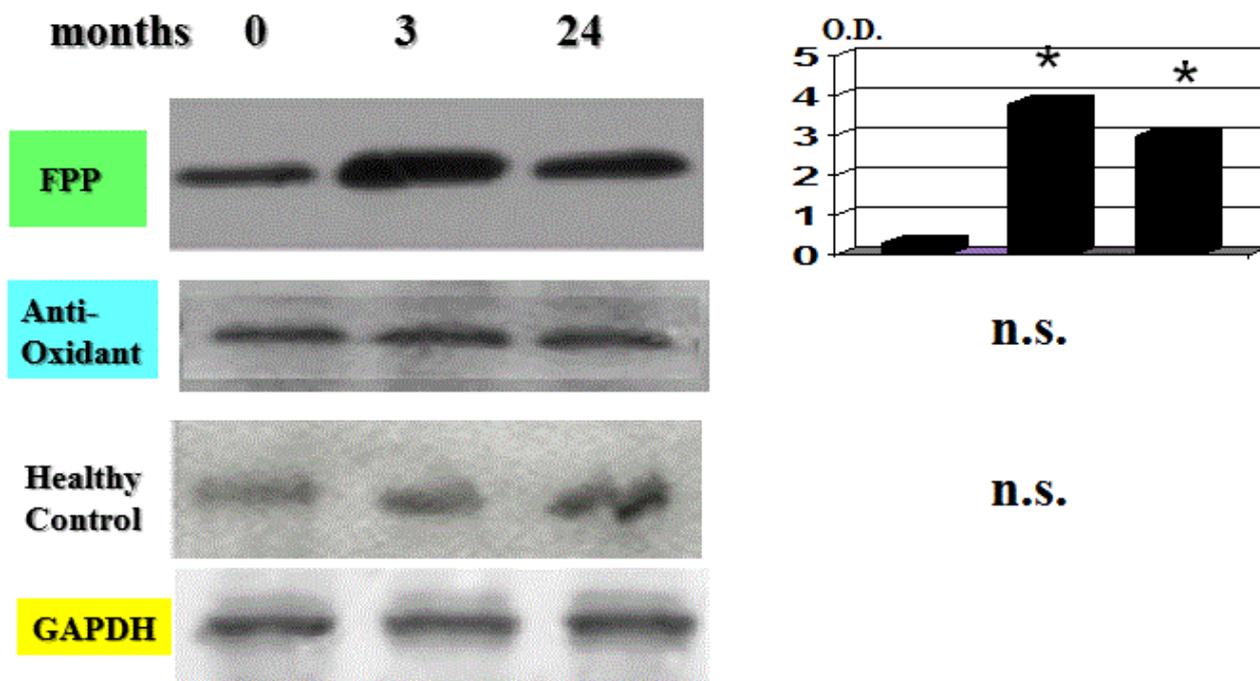
**Figure 3:** Variation of plasma level of Cyclophillin-A during treatment with the two nutraceuticals formulas. $^*$p<0.01 vs. baseline values; $^{**}$p<0.01 vs. FPP-treated group.
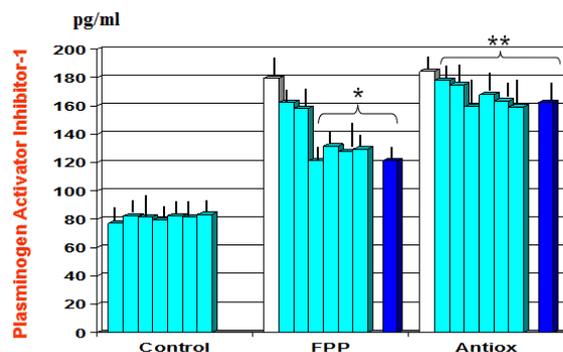


**Figure 4:** Variation of plasma level of plasminogen-activator inhibitor-1 during treatment with the two nutraceuticals formulas. $^*$p<0.01 vs. baseline values; $^{**}$p<0.01 vs. FPP-treated group.



**Figure 5:** Cyclophillin-A gene expression at baseline and during 24 months follow up under treatment with two different nutraceuticals. Only FPP was effective in upregulating this parameter ($^*$p<0.05 vs. baseline and vs. Antioxidant cocktail).

| Months | | Normal range | FPP | AntiOx mixture | Chelator |
|---|---|---|---|---|---|
| Mercury<br>mg/kg dry wt. | 21<br>24 | <0.5 | (0.3 ± 0.2)<br>0.4 ± 0.3 | (0.4 ± 0.1)<br>0.4 ± 0.2 | (0.3 ± 0.2)<br>1.1 ± 0.3* |
| Cadmium<br>mg/kg dry wt. | 21<br>24 | <0.5 | (0.2 ± 0.3)<br>0,5 ± 0.14 | (0.4 ± 0.3)<br>0.5 ± 0.12 | (0.3 ± 0.1)<br>1.4 ± 0.31* |
| Lead<br>mg/kg dry wt. | 21<br>24 | <0.5 | (0.2 ± 0.1)<br>0.2 ± 0.6 | (0.2 ± 0.12)<br>0.3 ± 0.09 | (0.3 ± 0.02)<br>1.1 ± 0.41* |
| Arsenicum<br>mg/kg dry wt. | 21<br>24 | <0.3 | (0.1 ± 0.04)<br>0.09 ± 0.032 | (0.1 ± 0.08)<br>0.1 ± 0.03 | (0.3 ± 0.04)<br>0.2 ± 0.12 |
| Beryllium<br>mg/kg dry wt. | 21<br>24 | <0.009 | (0.004 ± 0.003)<br>0.009 ± 0.004 | (0.007 ± 0.004)<br>0.004 ± 0.003 | (0.009 ± 0.004)<br>0.015 ± 0.002* |
| Nickel<br>mg/kg dry wt. | 21<br>24 | <8 | (5.8 ± 0.8)<br>5.6 ± 0.3 | (6.4 ± 1.2)<br>6.2 ± 1.3 | (6.1 ± 0.14)<br>6.9 ± 0.38 |

**Table 3:** Faecal heavy metal test: Effect of nutraceuticals with and without selected chelator. In brackets: baseline values corresponding to time 21 months and 24 month observation period of the trial. *$p < 0.05$ vs. baseline and vs. antioxidant cocktail.

On the other hand, a one-year consumption of a nutraceutical rich in resveratrol, one of the ingredient of our antioxidant cocktail, had shown to improve PAI-1, but CyPA and oxidised lipid markers were not tested [48]. A vascular component of neurodegenerative disease pathophysiology is credited by several epidemiological studies [49] and Oh et al. [50] has shown that patients with MCI and AD had significantly higher plasma PAI-1 levels, These appeared to correlate with cognitive function thus being a potentially early biomarker for AD. The upregulation of PAI-1 has also been suggested to explain insufficient BDNF neurotrophic support and increased neurodegeneration while suppressing PAI-1 expression/activity increases Aβ degradation [51]. To the vascular hypothesis is likely to cooperate also an increased CyPA which may affect endothelial cells and vascular smooth muscle cells [52,53]. Whatever the main mechanisms, it seems that by matching the metabolic profiling and AD, there might be three subtypes; an inflammatory one, a non-inflammatory type and a zinc deficiency-associated type affecting younger population [54]. In a prior study using a sturgeon eggs-derived compound of putative anti-inflammatory effect (Caviarlieri, Swiss cap, Switzerland) we had shown to increase circulating level of BDNF in healthy occupationally-stressed individuals [55]. However, internal and follow up data showed that this was a rather short-lived effect and disappointingly unaffecting PAI-1, OxLDL, Cyp-A or proBDNF. So, its initially suggested neuroprotective effect *in vitro* (but not at brain gene expression level in animals) had to be abandoned on clinical ground by our group.

On the other hand, the downregulating effect played by FPP on CyPA gene expression, but not from the antioxidant control, may be of relevance, when considering that it has become clear that a hyperglicemia and oxidative stress milieu may stimulate this molecule [56] which, on its turn, drives the ox-LDL-mediated differentiation and activation of monocytes to foam cells [57,58].

Heavy metals gut clearance was not affected by any of the nutraceuticals. However, the chelator, by itself remarkably increased fecal gut discharge of heavy metals but only in those patients with abnormal baseline values. The addition of a chelator to the control

nutraceuticals didn't prove to yield better results while the addition to FPP showed a trend increase of fecal discharge of cadmium (p<0.07 vs. oral chelator alone, n.s.).

The identification and application of novel biomarkers in nutritional and life-style interventional plans may foster healthy longevity and beneficially affect the occurrence of concomitant illnesses. Our preliminary data suggest that FPP might play a noteworthy role within a wider and comprehensive preventive medicine strategy plan for impending metabolic diseases which are potential co-factors of neurodegenerative disease. Environmental neurotoxins disrupt protein processing as shown in experimental studies [26-28] while effective heavy metals chelators remain a further additive avenue to possibly pursue in selected cases.

## Conclusion

As overall platform, virtuous dietary regimen such as the nutritional model of Mediterranean diet still maintains its importance to counter fight the cellular events involved in the atherosclerosis and neurodegenerative process [59,60]. However, given these promising results, longer studies addressing the issue of specific long term evidence-based nutraceutical implementations are highly awaited. Lastly, most recent research work points out the crucial importance of mitochondrial dynamics in metabolic and neurodegenerative diseases [61]. Indeed, exposure of environmental toxin, high-calorie intake, glucolipotoxicity brings about dysfunctional mitochondria which perpetuate ROS spilling over. Interestingly, the work of Collard et al. [62] in diabetic rats has shown how FPP optimised mitochondrial energetics. Such "cellular energetic" hypothesis as one of the possible FPP mechanisms also crossing the blood-brain-barrier has been recently envisaged by Hayashi Y. (in: Mantello A, Catanzaro C, He F, Cuffari B, Bissi L, Milazzo M, Lorenzetti A. Marotta F. Novel Nutrigenomics Avenues in Nutraceuticals Use: The Current Status of Fermented Papaya Preparation–Ed. E. Aguilar, Bioactive compounds: At the frontier between nutrition and pharmacology, 2016; 94-119).

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