

Effects of fish-derived lipoprotein extracts on activation markers, Fas expression and apoptosis in peripheral blood lymphocytes

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Received 16 July 2004; received in revised form 7 September 2004; accepted 20 September 2004

Abstract

Several factors may influence numbers and function of peripheral blood lymphocytes (PBLs) by different processes. We conducted this study to evaluate the effect of E-CAB-94011[®] and E-JUR-94013[®], two marine fish extracts from *S. scombrus* and *T. trachurus*, respectively, on in vitro PBLs activation and on the expression and functionality of Fas, a cell surface molecule that plays a central role in immune homeostasis and cytotoxic activity. PBLs from 24 healthy volunteers were isolated and flow cytometry was performed to measure the state of activation, Fas expression and apoptosis of PBLs. Functionality of Fas was tested by assessing apoptosis after incubation of isolated lymphocytes with agonistic anti-Fas antibodies in blood samples treated with both E-CAB-94011[®] and E-JUR-94013[®]. Studies on the lymphocyte cell marker suggest a clear immune activation as measured by the increased levels of CD25, CD8, CD38, CD19 and HLA-DR in vitro expression on lymphocytes treated with both extracts. In addition, a significant reduction in the percentages of apoptotic CD19⁺CD38⁺ double positive lymphocytes could be demonstrated in the treated samples with respect to controls ($p < 0.05$). Therefore the present results indicate that both E-CAB-94011[®] and E-JUR-94013[®] in vitro are powerful immunoregulatory, increasing immune surveillance.

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Keywords: Fas; Immune system; CD antigens; E-CAB-94011[®]; E-JUR-94013[®]

1. Introduction

The immune system represents a sophisticated machinery for protection against damage from invading

bacteria, viruses, and cancers. One of the most common immune weakeners is malnutrition, the commonest cause of immunodeficiency worldwide [1], and nutritional deficiencies are seen in at least one-third of the elderly in industrialized countries [2–5]. Stress, a major assault on immune health [6] as well as overeating and obesity, now epidemic in the Western world, also decrease immune function [7,8]. Perhaps the most inevitable weakener of our immune

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function is ageing itself. Ageing, which is associated with a decline in immune function that leads to an increased incidence of infection, cancer, and autoimmune disease, is known to bring about adverse changes in almost every aspect of our immune power, as alterations in both T and B cell functions [9–11].

The task at present is to define biomarkers (immunologic indices) that can predict resistance to infection and other illnesses associated with poor immune function with reasonable accuracy. We do know that the older we get, and the poorer our diet, the more susceptible we are to infection, tumor, and immune dysfunction. Free radicals have been linked to the immune system damage that accompanies normal aging [12–14]. Vitamins play a critical role along with trace elements in maintaining the human immune system [15]. Several herbs have demonstrated the ability to enhance and strengthen the immune system. These include echinacea, and ginseng and astragalus extracts [16,17]. Other nutrients that have shown a positive effect on immune function are lactoferrin [18,19], whey extract [20], L-carnitine and CoQ10 [21]. Useful hormones and extracts are melatonin, DHEA, glucocorticoids and estrogens [22–24].

Fish contains various antioxidants for protection of their unsaturated lipids and other nutrients from reactive oxygen species [25]. Oxidative protection of fish includes enzymes, various water-soluble and lipid soluble antioxidant systems. The function of these antioxidants is to control pro-oxidants, scavenging free radicals and inactivating reactive oxygen species as well [26].

Recently, research on antioxidants has gained much interest due to increased evidence of the importance of antioxidants in human health [27]. This interest has brought about considerable research on defining and finding components with antioxidative activity in humans and in biological systems. Relatively few studies on the various antioxidants in fish are currently available. Studies on the interaction between the diverse endogenous antioxidants in fish are also very few and may be difficult to rationalize because of the multiplicity of the antioxidant system in fish. However, the occurrence of the most abundant endogenous antioxidants found in fish such as tocopherols and carotenoids is fairly well established. The relative concentration of antioxidants varies with

the fish species as well as the type of fish muscle tissue [28]. The quantity of these antioxidants may also fluctuate with storage time and handling post mortem. Control of lipid oxidation in fish involves utilizing processing and storage techniques that do not greatly decrease the activity of endogenous antioxidants.

Because antioxidants play a key role in minimizing damage to cells, such as those that make up the immune system, recent research examined the benefits of certain antioxidants on the immune response of dogs and cats [29,30]. The results of these studies indicated that antioxidants are important in helping dogs and cats maintain a healthy immune system. Since the T cell response, the T cell dependent antibody response, the T and B cell counts, the natural killer (NK) activity vary, the mechanisms underlying these changes in immune function and peripheral blood lymphocyte (PBL) distribution contribute to the understanding of the direct effect caused by both pharmacological and nutritional compounds.

As with many animal products, E-CAB-94011® and E-JUR-94013® fish extracts contain water, proteins and other nitrogenous compounds, lipids, minerals and vitamins. Proteins comprise structural proteins (actin, myosin, tropomyosin and actomyosin), sarcoplasmic proteins (myoalbumin, globulin and enzymes) and connective tissue proteins (collagen). Due to the freezing/drying transformation process used for their production, E-CAB-94011® and E-JUR-94013® are able to maintain their chemical structure and, like milk, eggs and mammalian meat proteins, have a very high biological value. As well as being a rich source of high quality protein, they have a relatively high energy content and are rich in important minerals such as phosphorus, in B vitamins, in Eicosapentaenoic Acid (EPA) and in Docosahexaenoic Acid (DHA). EPA, a member of the Omega-3 fatty acid family is required for the production of prostaglandins which control blood clotting and other arterial functions [31–33] and DHA, a major component of the human brain tissues and retinal tissues of the eyes, participates in the transmission of nerve impulses in the nervous system [34]. Both DHA and EPA may help prevent heart disease and atherosclerosis by lowering triglyceride levels, raising HDL cholesterol and also reduce the risk of stroke and cardiac arrhythmias [35].

The main focus of our work was first to evaluate how the expression of molecules, associated with activation or “memory” status in lymphocytes, was modified after E-CAB-94011® and E-JUR-94013® in vitro stimulation, and second to understand how both extracts regulated the expression of Fas, a membrane receptor, that plays a central role in cellular immune homeostasis and mediates essential signals in activated mature lymphocytes.

2. Materials and methods

2.1. Extract preparation

E-CAB-94011® and E-JUR-94013® are two marine fish extracts obtained through biotechnological methods and treatment of specific components of the fish [36]. The main chemical composition of E-CAB-94011® is: 20% fat, 74% nitrogenous material, 4% ash and 2–3% moisture, while in E-JUR-94013® a 78% of nitrogenous material, a 16% of fat, a 4% ash and 2–3% of moisture, was quantified. More than 50% of fatty substances are unsaturated fatty acids (Arachidonic, Gadoleic, Linoleic, Linoleic, and Clupanodonic acids) and 8% of more complex lipid esters such as sterols. In the sterols group, more than 99% was represented by cholesterol. Extracts were maintained at –40 °C to prevent fat oxidation. Each extract was used at a final concentration of 5 mg/ml in complete medium (RPMI 1640+2 mM glutamin+60 µg/ml gentamycin+5% human pool serum).

2.2. Lymphocyte isolation

Five milliliters blood samples from healthy volunteers were drawn into a EDTA-coated. The blood were diluted with an equal volume of phosphate-buffered saline (PBS), overlaid with 5 ml of sodium metrizoate-Ficoll for human lymphocytes, and centrifuged at 1200 rpm for 30 min. The fraction consisting of the peripheral blood mononuclear cells (PBMCs) was washed with PBS three times, and maintained in static culture in RPMI 1640 medium supplemented with 5% human pool serum at 37 °C in an atmosphere of 5% CO₂. Four hours later, the plastic-adherent cells were removed, and PBMCs (5×10⁵ cells/ml) consisting mostly of lymphocytes were isolated.

2.3. PHA activation

To induce lymphocyte division, a polyclonal mitogen, phytohemagglutinin (PHA), was added to the PBMCs, which were incubated for 2 days. PHA (5–10 µg/ml) was administered to PBMCs consisting mostly of lymphocytes and, after incubation for 48 h, PHA treated samples were used as positive/activated lymphocyte controls and stained with monoclonal antibodies.

2.4. E-CAB-94011® and E-JUR-94013® treatments and cell viability

To test the effects of E-CAB-94011® and E-JUR-94013® on lymphocytes, 5 mg/ml of each extract were added to the PBMCs, which were incubated for 2 days. After incubation for 48 h, treated samples were washed with PBS, centrifuged, resuspended in PBS and stained with monoclonal antibodies. To measure viability, a small sample of the cell suspensions were diluted 1:5 in 0.4% (w/v Trypan blue). A 20 µl of suspensions was added to a hemacytometer chamber and an inverted microscope with a 100× magnification was used to perform cell counts. The number of cells per ml and total number of cells was determined by using the following calculations:

$$\text{cells/ml} = \frac{\# \text{cells counted} \times 10^4 \times \text{dilution factor}}{\# \text{squares counted}}$$

$$\text{total cells} = \text{cells/ml}$$

$$\times \text{vol. of original cell suspension}$$

The percentage of viable cells was calculated using the following formula:

$$\% \text{Viability} = \frac{\# \text{Viable Cells Counted} \times 100}{\text{Total \# Cells Counted}}$$

2.5. Cell staining, FACS analysis and apoptosis assay

For surface staining the following conjugated monoclonal antibodies (mAb) were used: (CD3, CD4, CD8, CD19, CD25 and HLA-DR). In brief, 100 µl of blood was added to tubes and stained with the monoclonal antibody specific for lymphocyte marker during a 45-min incubation on ice in the dark.

Following staining, red blood cells were lysed using diluted (1×) FACS lysing solution. Immediately after a 10 min incubation with lysing solution, tubes were centrifuged at 300×*g* for 5 min. Supernatants were aspirated leaving approximately 50 µl of residual fluid in the tube to avoid disturbing the pellet. Cells were gently resuspended in the residual fluid, washed with 2 ml of phosphate buffered saline and then centrifuged at 200×*g* for 5 min. Washing was repeated twice. After the addition of 222.5 µl binding buffer (10 mM hepes pH=7.4, 140 mM NaCl, 5 mM CaCl₂), 25 µl propidium iodide (10 µg/ml) and 2.5 µl FITC-labeled annexin V diluted 1:50 were added. Immediately after incubation for 10 min on ice in the dark, cells were analyzed using the flow cytometer (FACScan) after gating lymphocyte characteristics using both forward and sideward scatter. For optimal analysis only those samples were included in which more than 500 cells of the respective subsets were counted. CalIBRITE beads and AutoComp software were used for setting the photomultiplier tube voltages, the fluorescence compensation and checking instrument sensitivity prior to analysis. Percentages of cells staining positive for a particular marker were compared between groups. In addition, we compared mean fluorescence intensity (MFI) of staining between both groups. MFI represents the intensity of the signal on those cells positively staining for the respective marker. All analyses were performed consistently by the same person.

2.6. Apoptosis induction

To analyze whether the Fas-mediated pathway of apoptosis induction was functionally affected by E-CAB-94011® and E-JUR-94013® treatments, lymphocytes from 10 healthy subjects were incubated with an agonistic anti-Fas mAb. After isolation (t0), cells were resuspended in complete medium with and without the addition of 5 µg/ml anti-Fas. Subsequently, cells were cultured for 24 h (t1). Both immediately after isolation (spontaneous apoptosis) and after 24 h of culture (induced apoptosis) were analysed for apoptosis assessment. To indirect monitor PS translocation after E-CAB-94011® and E-JUR-94013® treatment, PBLs from 10 healthy subjects were incubated with anti-Fas for 0 to 24 h, stained with annexin V-PE, a 35–36 kDa, calcium-dependent, phospholipid-binding protein with a high

affinity for PS and Active Caspase-3 FITC monoclonal antibodies.

2.7. Statistical analysis

Differences between groups were evaluated using the Student's *t*-test or Mann–Whitney *U*-test when appropriate. Paired samples were analysed separately using the Wilcoxon rank sum test. Spearman's rank correlation was applied for detecting correlations between different study parameters. A *p* value less than 0.05 was considered statistically significant.

3. Results

PBLs obtained from five normal volunteers were treated with different concentrations of both E-CAB-94011® and E-JUR-94013® to perform preliminary toxicity tests. Cytotoxicity was assessed by the trypan blue dye exclusion test after 24, 48, 72 and 96 h in culture. As it can be observed in Table 1, no cytotoxic effects were observed with both CAB-94011® and E-JUR-94013® extracts. Concentrations ranging from 1 to 50 mg/ml did not show any sign of cytotoxicity as compared to untreated cultures.

Table 1
Cytotoxic effects of E-CAB-94011® and E-JUR-94013® on PBLs

	24 h	48 h	72 h	96 h
Untreated	2.5±0.17	2.6±0.15	2.9±0.18	3.3±0.21
E-CAB-94011® (1 mg/ml)	2.6±0.11	2.8±0.14	2.9±0.22	3.2±0.22
E-CAB-94011® (5 mg/ml)	2.7±0.15	2.5±0.11	3.1±0.15	3.5±0.19
E-CAB-94011® (25 mg/ml)	2.5±0.17	2.8±0.13	3.2±0.19	3.2±0.21
E-CAB-94011® (50 mg/ml)	2.7±0.11	2.9±0.17	3.2±0.21	3.3±0.16
E-JUR-94013® (1 mg/ml)	2.5±0.14	2.6±0.16	2.9±0.18	3.1±0.21
E-JUR-94013® (5 mg/ml)	2.6±0.15	2.7±0.18	3.2±0.19	3.3±0.22
E-JUR-94013® (25 mg/ml)	2.5±0.21	2.8±0.19	2.9±0.16	3.4±0.21
E-JUR-94013® (50 mg/ml)	2.7±0.19	2.9±0.14	3.0±0.21	3.2±0.32

Cytotoxicity was assessed by the trypan blue dye exclusion test up to 96 h in culture. The data represent the mean of three different experiments with S.D.

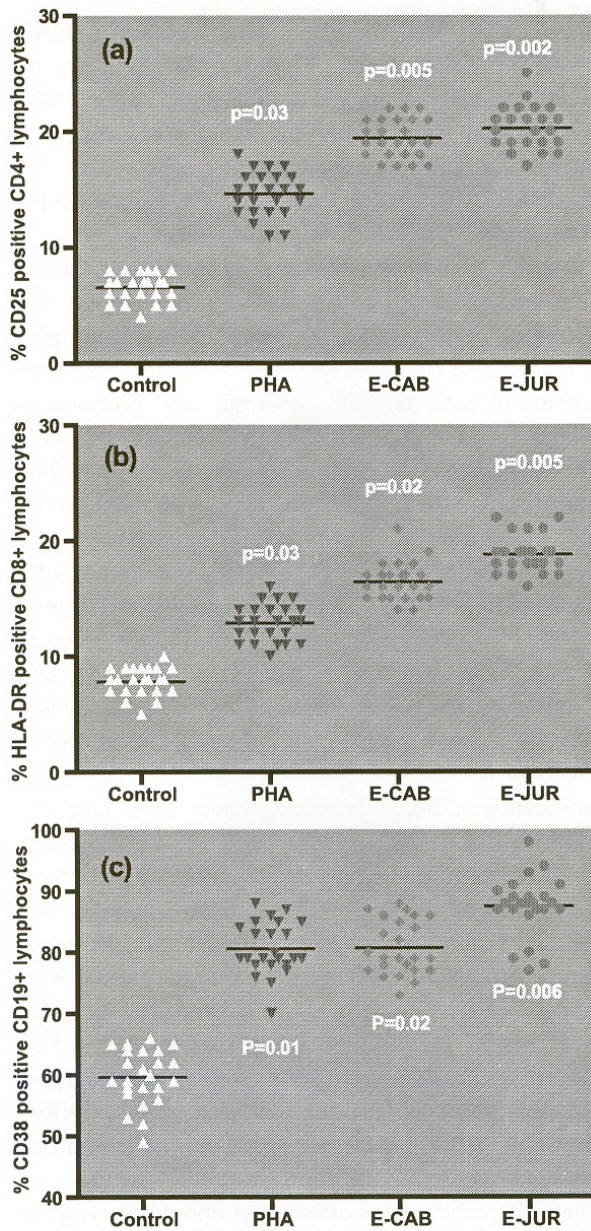


Fig. 1. Percentages of activated lymphocytes of the subpopulations analysed after E-CAB-94011 and E-JUR-94013 in vitro treatment. (a) Percentage of CD4⁺ cells expressing the activation marker CD25. (b) Percentage of CD8⁺ cells expressing the activation marker HLA-DR. (c) Percentage of CD19⁺ cells expressing the activation marker CD38. Horizontal lines denote the median. Only results of those samples are shown in which the number of cells measured exceeded 500.

Twenty-four blood samples were used to study the effects of E-CAB-94011[®] and E-JUR-94013[®] on in vitro activation of lymphocytes. CD4⁺ lymphocytes

showed signs of increased activation reflected by increased expression of CD25, the interleukin-2 receptor ($p=0.005$, $p=0.002$ versus controls, Fig. 1a). Percentages of the CD8⁺ T-lymphocytes expressing the activation marker HLA-DR tended to be higher after treatment ($p=0.02$, $p=0.005$, versus control, Fig. 1b).

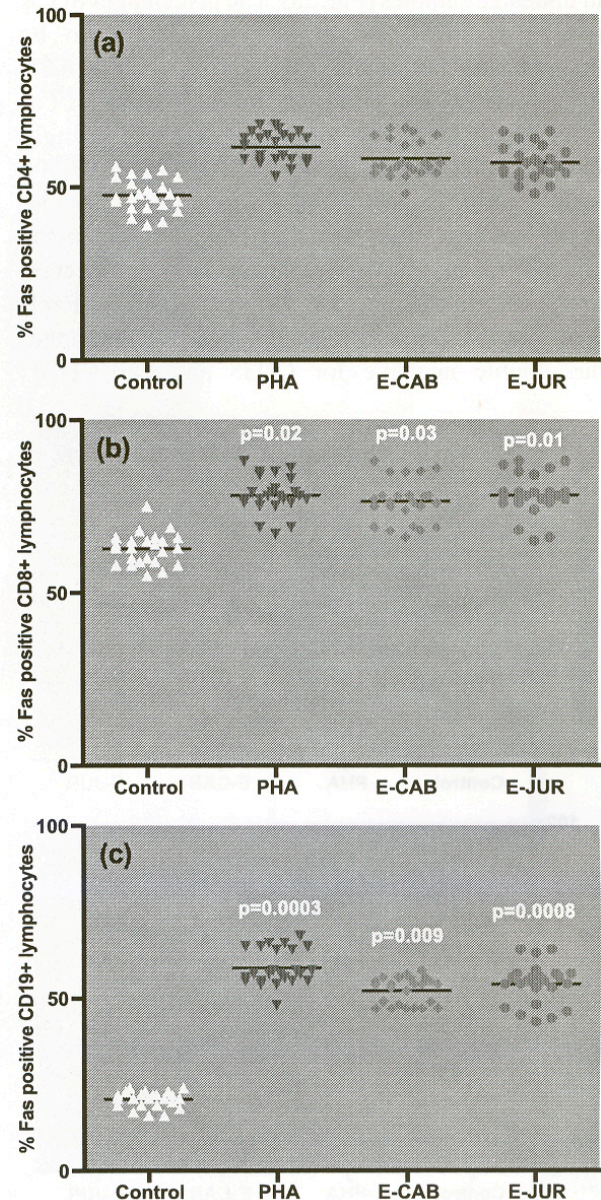


Fig. 2. Fas (CD95) expression on CD4⁺ (panel a), CD8⁺ (panel b) and CD19⁺-lymphocytes (panel c) after E-CAB-94011 and E-JUR-94013 in vitro treatment. Horizontal lines denote the median. Only results of those samples are shown in which the number of cells measured exceeded 500.

Percentages of CD19⁺ B-lymphocytes expressing the activation marker CD38 were also increased after treatment compared to untreated controls ($p=0.02$, $p=0.006$, versus control, Fig. 1c). Fas expression was measured on CD4⁺, CD8⁺, as well as CD19⁺ lymphocytes (Fig. 2). Fas expression on CD4⁺ T-lymphocytes did not show significant differences between treated and untreated samples (Fig. 2a). The percentages of Fas expressing CD8⁺ T-lymphocytes tended to be increased after both treatments ($p=0.03$ and $p=0.01$, versus controls, 2b). However, the percentage of Fas expressing CD19⁺ B-lymphocytes was strongly increased after treatment ($p=0.009$ and $p=0.0008$ versus control, respectively). To investigate whether Fas expression was associated with lymphocyte activation we analysed Fas expression in conjunction with CD38 expression as a membrane marker for B cell activation (Fig. 3). The percentage of lymphocytes double negative for CD38 and Fas CD19⁺

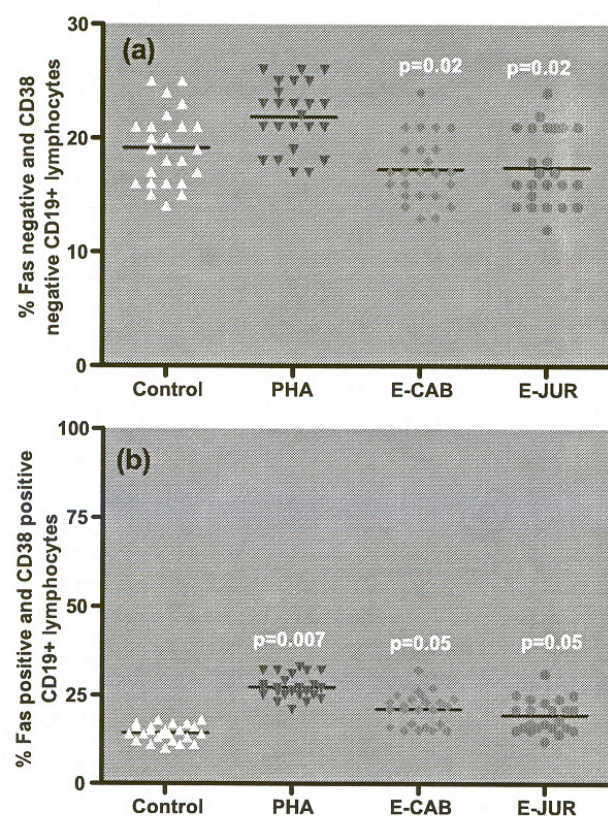


Fig. 3. Fas (CD95) expression on CD19⁺ B-lymphocytes in relation to the expression of the activation marker CD38 after E-CAB-94011 and E-JUR-94013 in vitro treatment. Horizontal lines denote the median. Only results of those samples are shown in which the number of cells measured exceeded 500.

lymphocytes was reduced, compared to controls ($p=0.02$ in both cases, Fig. 3a). In contrast, the percentage of lymphocyte double positive for CD38 and Fas was significantly increased with respect to control ($p=0.05$ in both cases, Fig. 3b). No correlation was found between the percentages Fas expressing CD4⁺- or CD8⁺-T-lymphocytes.

Results of anti-Fas induced apoptosis of CD19⁺ cells are shown in Fig. 4. The percentage Fas expressing lymphocytes increased in medium after 24 h; increase of Fas expression occurred also after incubation with anti-Fas, though less pronounced (data not shown). Percentages of apoptotic B cells were low directly after isolation, increased after 24 h incubation in medium alone, and, compared to the latter value, reached significantly higher percentages after incubation with anti-Fas, demonstrating the functionality of the Fas-induced apoptotic pathway (Fig. 4). Untreated samples were primarily negative for both markers, positive peaks were apparent at 1 h, and the percentages of positive cells increased over the 24-h treatment. After 24 h of apoptosis induction in B cells, percentages of apoptosis significantly differed between treated (t1b and t1c) and untreated (t1a) lymphocytes (Fig. 4), indicating the capacity of both extracts to reduce significantly the proportion of apoptotic B cells ($p=0.03$ and $p=0.02$, respectively). Percentages of apoptosis reached in T cells after apoptosis induction were comparable between treated and untreated lymphocytes after 24 h (data not shown). As we found Fas-induced apoptosis significant different in treated and untreated lymphocytes, we wondered whether differences in percentages of spontaneous apoptotic lymphocytes could be demonstrated in the two groups. Percentages of circulating apoptotic lymphocytes were assessed by annexin V staining. Although percentages of apoptotic CD19⁺ cells, CD4⁺ cells and CD8⁺ T cells were higher in the group of treated lymphocytes, differences were not statistically significant (Table 2).

4. Discussion

In vitro assays of cellular function contribute significantly to a better understanding of the immune response and mechanisms of host defense. Lympho-

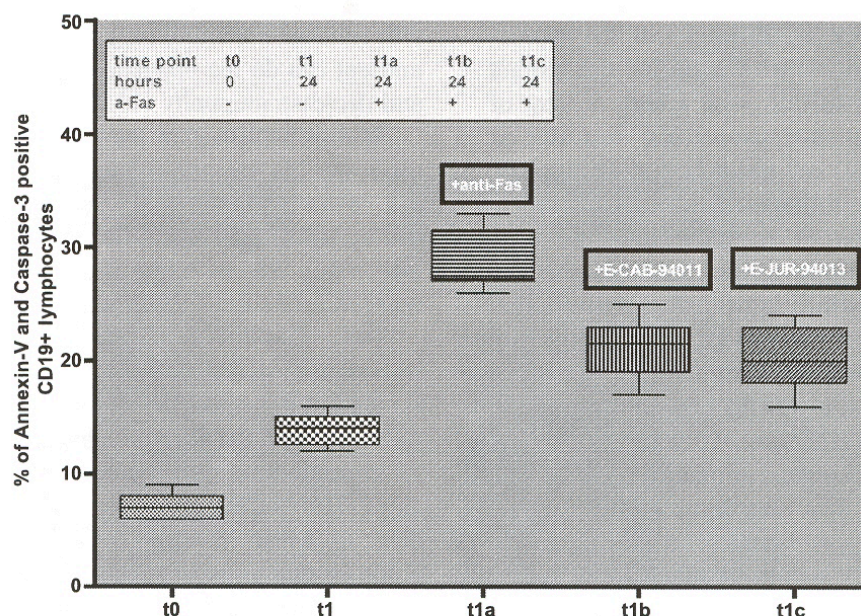


Fig. 4. Percentages of apoptotic annexin-V PE and Caspase-3 FITC CD19⁺ lymphocytes analysed at: t0, immediately after lymphocytes isolation; t1, after 24 h incubation in medium; t1a, after 24 h incubation with anti-Fas following incubation with anti-Fas antibodies; t1b, after 24 h incubation with anti-Fas following incubation with anti-Fas antibodies and E-CAB-94011; t1c, after 24 h incubation with anti-Fas following incubation with anti-Fas antibodies and E-JUR-94013. Apoptotic cells are depicted as those staining positive with both annexin V and Caspase-3.

cytes, monocytes, granulocytes and dendritic cells are the major cellular components of the immune system and play important roles in host defense. Each cell type expresses a unique phenotype pattern of surface molecules/receptors that may participate in biological mechanisms during the immune response. Targeted therapies that make use of the immune system and address selectively the individual target molecules or

cellular pathways that play a role in carcinogenesis came long after the discovery and first use of more general immunostimulatory agents such as staphylococcal enterotoxins and staphylococcal superantigens [37] or cytokines [38,39].

T cells express T-cell receptor molecules that are noncovalently associated with the CD3 complex. Interaction of antigens presented via major histocompatibility complex (MHC) molecules with TCR activates T cells and induces T-cell proliferation and differentiation. The executives of specific cellular immunity are activated CD8⁺ killer T cells and CD4⁺ helper T cells, which bear T cell receptors that can recognize cells expressing MHC molecules on their surface. Not only does every positive reconnaissance of a host tissue cell by a killer T cell require MHC Class I expression, but recognition necessarily depends on short peptides bound by those MHC Class I molecules. Short peptides allow killer T cells to discriminate between non-rendered cells with an unchanged proteome and altered cells, which express viral proteins, mutated self-proteins or self-proteins that appear at increased expressed levels in association with a certain state of disease.

Table 2
Percentages of total lymphocytes and lymphocyte subsets staining with annexin V in the treated and untreated groups

	E-CAB- treated (n=10)	<i>p</i> value	E-JUR- treated (n=10)	<i>p</i> value	Untreated (n=10)
% Lymphocytes annexin V ⁺	5.5±4.3	0.76	5.2±3.2	0.77	4.7±3.5
% CD4 ⁺ annexin V ⁺	3.3±3.7	0.28	3.9±3.5	0.59	2.9±2.5
% CD8 ⁺ annexin V ⁺	4.8±4.4	0.39	3.8±42.3	0.45	4.5±3.8
% CD19 ⁺ annexin V ⁺	8.6±5.8	0.66	6.9±4.9	0.81	7.7±5.9

Data are means±S.D. *p* values were calculated using the Mann-Whitney *U*-test (two-tailed).

In a previous work we showed that the supplementation with E-SAR-94010® a marine fish extract obtained from *S. pilchardus*, with a high content of long-chain polyunsaturated fatty acids, was able to modulate T_H1 and T_H2 cell generation, their cytokine production, and cell proliferation and to improve the immunological status of pregnant rats and their sucking pups [40]. These results indicated that the secondary response, which is typically more specific, rapid and of a greater magnitude, was enhanced by gestational E-SAR-94010® supplementation. Based on these observations, it was possible to demonstrate that the incorporation in the diet of this fish extract was able to influence the postnatal immune response of the offspring and these data suggested that substantial amounts of fatty acids and lipoproteins were present in both rat breast milk as well as at the mucosal level.

Recent studies in humans and in experimental animals indicated that the immunomodulatory effects of fish oils depend on synergistic effects of at least EPA and DHA [41,42]. Nutritional modification of cellular functions by dietary lipids with a balanced ratio of ω -6 and ω -3 fatty acids offers an attractive avenue to correct, modify and/or prevent many pathophysiological processes in health and disease state and to reduce toxicity of drugs in many patients. The mediation of such effects is thought to be primarily achieved through alterations of cellular membranes composition and other endogenous lipid stores which may modify the functional activity of various receptors on plasma membranes. Many investigators have demonstrated that ω -6 (n-6) and ω -3 (n-3) PUFAs including linoleic acid (LA), gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA), arachidonic acid (AA), alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) inhibit growth and are cytotoxic to cancer cells in vitro [43,44], that the effects are associated with the production of lipid peroxides and aldehydes [45], and that the cytotoxicity of the added PUFAs is reduced by the addition of antioxidants. Studies with laboratory animals have also demonstrated that feeding a diet containing peroxidation products of fish oil reduces tumor growth, and that the effect is reduced by administering antioxidants [46].

From the above, it can be seen that a strong immune system is dependent on a good foundation of nutrition. There is no single nutrient that, by itself,

will enhance immunity [47]. In fact, too much of one nutrient can do the opposite of what one might want and decrease immunity [13,48].

Studies published in the 1980s and 1990s clearly show specific immune-enhancing effects of the proper use of nutritional supplements, proteins, hormones, and certain drugs. Micronutrients are now known to play a key role in many of the metabolic processes that promote survival from critical illnesses [49,50].

The goal of this investigation was to study the effect of both E-CAB-94011® and E-JUR-94013® extracts on the in vitro activation of immune cells. Regarding the effect on T-lymphocyte subpopulations, significant differences were observed on CD8, CD25, HLA-DR and CD19⁺CD38⁺ double positive cells after treatment (Fig. 1), which indicates that both extracts act on a common step in the activation of these cell types, that is, a common central step is probably required for the activation of these subpopulations, such as the activation of transcription factors. To determine the possible toxic effect of both extracts in whole blood samples, we quantified the amount of apoptotic cells after overnight incubation. Surprisingly, the tested extracts did not show any sign of toxicity and the expression of Fas on CD4⁺, CD8⁺ and CD19⁺ lymphocytes (Fig. 2) as well as on CD19⁺CD38⁺ and CD19⁺CD38⁺ (Fig. 3) was reduced comparing to activated positive controls. These results were unexpected considering the level of activation observed and one possible explanation for the observed results is that the lack of toxicity could be a consequence of the inhibition of transcription factors which directly act on the CD95 death receptor.

A measurable feature of apoptosis is the loss of membrane asymmetry. In normal cells, membrane phospholipids are distributed asymmetrically between the inner and outer leaflets of the plasma membrane. For example, phosphatidylserine (PS), an aminophospholipid, is normally present in the inner leaflet of the plasma membrane. Early in apoptosis, before the loss of membrane integrity, PS translocates from the inner to the outer leaflet of the plasma membrane, exposing it to the external cellular environment at the surface of the plasma membrane.

As cells function normally in the body, they produce damaged molecules, such as free radicals. These free radicals are highly unstable and steal components from other cellular molecules, such as fat, protein, or DNA, thereby spreading the damage. This

damage continues in a chain reaction, and entire cells soon become damaged and die. Antioxidants help prevent widespread cellular destruction by willingly donating components to stabilize free radicals. More importantly, antioxidants return to the surface of the cell to stabilize rather than damage other cellular components.

When there are not enough antioxidants to hold peroxidation in check, free radicals begin damaging healthy cells which, in turn, can lead to problems. For example, free radical damage to immune cells can lead to an increased risk of infections [51–53]. Since optimal T-cell activation requires multiple signals provided by recognition of peptide-major histocompatibility complex (MHC) proteins, by the T-cell receptor (TCR), and by interaction of T-cell co-stimulatory receptors with their ligands on antigen-presenting cells [54,55], it could be possible that some components, like small peptides, PUFAs, in combination with antioxidants of both E-CAB-94011® and E-JUR-94013® have a major role in the in vitro modulation of the expression of genes concerned with immune response as well as in the downregulation of the pathological mechanisms that tend to induce the expression of apoptotic genes.

The study of the potential ability of bioactive compound(s) present in both E-CAB-94011® and E-JUR-94013® to participate in cell activation, oxidative processes, immune responses, and inflammation is only starting, and more research must be done in order to understand the complete mechanism of their action. By the production of bioactive compounds through the use of biotechnological advanced processes, these findings will aid the development of new food-based products that help to prevent poor specific cellular immune responses.

References

- [1] Dabis F, Orne-Gliemann J, Perez F, Leroy V, Newell ML, Coutoudis A, et al. Working Group on Women and Child Health. Improving child health: the role of research. *BMJ* 2002;324:1444–7.
- [2] Lesourd B. Nutrition: a major factor influencing immunity in the elderly. *J Nutr Health Aging* 2004;8:28–37.
- [3] Ahluwalia N, Sun J, Krause D, Mastro A, Handte G. Immune function is impaired in iron-deficient, homebound, older women. *Am J Clin Nutr* 2004;79:516–21.
- [4] Callen B. Understanding nutritional health in older adults. A pilot study. *J Gerontol Nurs* 2004;30:36–43.
- [5] Clarke R, Grimley Evans J, Schneede J, Nexø E, Bates C, Fletcher A, et al. Vitamin B12 and folate deficiency in later life. *Age Ageing* 2004;33:34–41.
- [6] Gleeson M, Nieman DC, Pedersen BK. Exercise, nutrition and immune function. *J Sports Sci* 2004;22:115–25.
- [7] Lamas O, Marti A, Martinez JA. Obesity and immunocompetence. *Eur J Clin Nutr* 2002;56(Suppl. 3):S42–5.
- [8] Linton MF, Fazio S. Macrophages, inflammation, and atherosclerosis. *Int J Obes Relat Metab Disord* 2003;27(Suppl. 3):S35–40.
- [9] Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. *Nat Immunol* 2004;5:133–9.
- [10] Schindowski K, Kratzsch T, Peters J, Steiner B, Leutner S, Touchet N, et al. Impact of aging: sporadic, and genetic risk factors on vulnerability to apoptosis in Alzheimer's disease. *Neuromol Med* 2003;4:161–78.
- [11] Van der Put E, Sherwood EM, Blomberg BB, Riley RL. Aged mice exhibit distinct B cell precursor phenotypes differing in activation, proliferation and apoptosis. *Exp Gerontol* 2003;38:1137–47.
- [12] Haddad JJ. Redox and oxidant-mediated regulation of apoptosis signaling pathways: immuno-pharmaco-redox conception of oxidative siege versus cell death commitment. *Int Immunopharmacol* 2004;4:475–93.
- [13] De la Fuente M. Effects of antioxidants on immune system ageing. *Eur J Clin Nutr* 2002;56(Suppl. 3):S5–8.
- [14] Knight JA. The biochemistry of aging. *Adv Clin Chem* 2000;35:1–62.
- [15] Serafini M. Dietary vitamin E and T cell-mediated function in the elderly: effectiveness and mechanism of action. *Int J Dev Neurosci* 2000;18:401–10.
- [16] Block KI, Mead MN. Immune system effects of echinacea, ginseng, and astragalus: a review. *Integr Cancer Ther* 2003;2:247–67.
- [17] Ganju L, Karan D, Chanda S, Srivastava KK, Sawhney RC, Selvamurthy W. Immunomodulatory effects of agents of plant origin. *Biomed Pharmacother* 2003;57:296–300.
- [18] Dawes ME, Lakritz J, Tyler JW, Cockrell M, Marsh AE, Estes DM, et al. Effects of supplemental lactoferrin on serum lactoferrin and IgG concentrations and neutrophil oxidative metabolism in Holstein calves. *J Vet Intern Med* 2004;18:104–8.
- [19] Sfeir RM, Dubarry M, Boyaka PN, Rautureau M, Tome D. The mode of oral bovine lactoferrin administration influences mucosal and systemic immune responses in mice. *J Nutr* 2004;134:403–9.
- [20] Penttilä IA, Zhang MF, Bates E, Regester G, Read LC, Zola H. Immune modulation in suckling rat pups by a growth factor extract derived from milk whey. *J Dairy Res* 2001;68:587–99.
- [21] Arafa HM, Abd-Allah AR, El-Mahdy MA, Ramadan LA, Hamada FM. Immunomodulatory effects of L-carnitine and q10 in mouse spleen exposed to low-frequency high-intensity magnetic field. *Toxicology* 2003;187:171–81.
- [22] Carrillo-Vico A, Calvo JR, Abreu P, Lardone PJ, Garcia-Maurino S, Reiter RJ, et al. Evidence of melatonin synthesis by human lymphocytes and its physiological significance:

- possible role as intracrine, autocrine, and/or paracrine substance. *FASEB J* 2004;18:537–9.
- [23] Sacco M, Valenti G, Corvi Mora P, Wu FC, Ray DW. DHEA, a selective glucocorticoid receptor antagonist: its role in immune system regulation and metabolism. *J Endocrinol Invest* 2002;25:81–2.
- [24] Dietrich JB. Endothelial cells of the blood–brain barrier: a target for glucocorticoids and estrogens? *Front Biosci* 2004;9:684–93.
- [25] Bragadottir M, Palmadottir H, Kristbergsson K. Composition and chemical changes during storage of fish meal from Capelin (*Mallotus villosus*). *J Agric Food Chem* 2004;52:1572–80.
- [26] Dautremepuits C, Betoulle S, Vernet G. Stimulation of antioxidant enzymes levels in carp (*Cyprinus carpio* L.) infected by *Ptychobothrium* sp. (Cestoda). *Fish Shellfish Immunol* 2003;15:467–71.
- [27] Vitaglione P, Fogliano V. Use of antioxidants to minimize the human health risk associated to mutagenic/carcinogenic heterocyclic amines in food. *J Chromatogr B Anal Technol Biomed Life Sci* 2004;802:189–99.
- [28] Passi S, Cataudella S, Di Marco P, De Simone F, Rastrelli L. Fatty acid composition and antioxidant levels in muscle tissue of different Mediterranean marine species of fish and shellfish. *J Agric Food Chem* 2002;50:7314–22.
- [29] Hendriks WH, Wu YB, Shields RG, Newcomb M, Rutherford T, Belay T, et al. Vitamin E requirement of adult cats increases slightly with high dietary intake of polyunsaturated fatty acids. *J Nutr* 2002;132:1613S–35S.
- [30] Hall JA, Tooley KA, Gradin JL, Jewell DE, Wander RC. Effects of dietary n-6 and n-3 fatty acids and vitamin E on the immune response of healthy geriatric dogs. *Am J Vet Res* 2003;64:762–72.
- [31] Moonen HJ, Dommels YE, van Zwam M, van Herwijnen MH, Kleinjans JC, Alink GM, et al. Effects of polyunsaturated fatty acids on prostaglandin synthesis and cyclooxygenase-mediated DNA adduct formation by heterocyclic aromatic amines in human adenocarcinoma colon cells. *Mol Carcinog* 2004;40:180–8.
- [32] Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids* 2001;36:1007–24.
- [33] Calder PC. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. *Lipids* 2003;38:343–52.
- [34] Wang X, Zhao X, Mao ZY, Wang XM, Liu ZL. Neuroprotective effect of docosahexaenoic acid on glutamate-induced cytotoxicity in rat hippocampal cultures. *NeuroReport* 2003;14:2457–61.
- [35] Wijendran V, Hayes KC. Dietary n-6 and n-3 fatty acid balance and cardiovascular health. *Annu Rev Nutr* 2004;24:597–615.
- [36] Lombardi VR, Fernandez-Novoa L, Corzo D, Zas R, Cacabelos R. Enhancement in immune function and growth using E-JUR-94013 supplementation. *Methods Find Exp Clin Pharmacol* 2002;24:573–8.
- [37] Baker MD, Acharya KR. Superantigens: structure function relationships. *Int J Med Microbiol* 2004;293:529–37.
- [38] Arnold J, de Boer EC, O'Donnell MA, Bohle A, Brandau S. Immunotherapy of experimental bladder cancer with recombinant BCG expressing interferon-gamma. *J Immunother* 2004;27:116–23.
- [39] Braun M, Vierling JM. The clinical and immunologic impact of using interferon and ribavirin in the immunosuppressed host. *Liver Transpl* 2003;9:S79–89.
- [40] Lombardi RM, Cagiao A, Fernández-Novoa L, Álvarez XA, Corzo MD, Zas R, et al. Short food supplementation effects of a fish derived extract on the immunological status of pregnant rats and their sucking pups. *Nutr Res* 2001;21:1425–34.
- [41] Kew S, Mesa MD, Tricon S, Buckley R, Minihane AM, Yaqoob P. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. *Am J Clin Nutr* 2004;79:674–81.
- [42] Kew S, Gibbons ES, Thies F, McNeill GP, Quinlan PT, Calder PC. The effect of feeding structured triacylglycerols enriched in eicosapentaenoic or docosahexaenoic acids on murine splenocyte fatty acid composition and leucocyte phagocytosis. *Br J Nutr* 2003;90:1071–80.
- [43] Akihisa T, Tokuda H, Ogata M, Ukiya M, Iizuka M, Suzuki T, et al. Cancer chemopreventive effects of polyunsaturated fatty acids. *Cancer Lett* 2004;205:9–13.
- [44] Singh R, Singh RK, Mahdi AA, Singh RK, Kumar A, Tripathi AK, et al. Circadian periodicity of plasma lipid peroxides and other anti-oxidants as putative markers in gynecological malignancies. *In Vivo* 2003;17:593–600.
- [45] Yousef MI, Kamel KI, Esmail AM, Baghdadi HH. Antioxidant activities and lipid lowering effects of isoflavone in male rabbits. *Food Chem Toxicol* 2004;42:1497–503.
- [46] Stoll BA. N-3 fatty acids and lipid peroxidation in breast cancer inhibition. *Br J Nutr* 2002;87:193–8.
- [47] Lopez-Varela S, Gonzalez-Gross M, Marcos A. Functional foods and the immune system: a review. *Eur J Clin Nutr* 2002;56(Suppl. 3):S29–33.
- [48] Beisel WR, Edelman R, Nauss K, Suskind RM. Single-nutrient effects on immunologic functions. Report of a workshop sponsored by the Department of Food and Nutrition and its nutrition advisory group of the American Medical Association. *JAMA* 1981;245(1):53–8.
- [49] Chandra RK, Kumari S. Nutrition and immunity: an overview. *J Nutr* 1994;124:1433S–5S.
- [50] Chandra RK. Nutrition and the immune system from birth to old age. *Eur J Clin Nutr* 2002;56:S73–6.
- [51] Chan J, Flynn J. The immunological aspects of latency in tuberculosis. *Clin Immunol* 2004;110:2–12.
- [52] Bhattacharyya J, Biswas S, Datta AG. Mode of action of endotoxin: role of free radicals and antioxidants. *Curr Med Chem* 2004;11:359–68.
- [53] Guzik TJ, Korb R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. *J Physiol Pharmacol* 2003;54:469–87.
- [54] Workman CJ, Cauley LS, Kim IJ, Blackman MA, Woodland DA, Vignali DA. Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *J Immunol* 2004;172:5450–5.
- [55] Mirshahidi S, Ferris LC, Sadegh-Nasseri S. The magnitude of TCR engagement is a critical predictor of T cell anergy or activation. *J Immunol* 2004;172:5346–55.

Novel Biotechnological Products from Natural Sources: Nutri/Pharmacogenomic Component

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Received date: October 31, 2016; Accepted date: November 1, 2016; Published date: November 7, 2016

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Editorial

A series of novel bioproducts obtained from natural sources (marine, animal, vegetal) have been discovered and characterized for the prevention and treatment of Neuro Degenerative Disorders (NDDs) and vascular pathology. Lipofuscins (LFs) are a new class of lipoproteins derived from the muscle of different fish species. Examples of LPs obtained from biomarine sources by means of non-denaturing biotechnological procedures include the following: E-JUR-94013 (DefenVid[®]), E-CAB-94011 (CabyMar[®]), E-Congerine-10423 (AntiGan[®]), E-SAR-94010 (LipoEsar[®]), and E-MHK-0103 (MineraXin[®]) [1-6]. Most effects of these novel bio products are genotype-dependent, showing specific nutrigenomic and pharmacogenomic profiles [7-10].

E-CAB-94011 is an LF obtained from the muscle of the species *Scombrus scombrus*, with anti-oxidant, anti-inflammatory, and bio-energizing properties, with potential utility in several medical conditions (anemia, debilitating disorders, alterations in growth and development, ROS generation, NDDs) [3].

E-Congerine-10423 is an LP extracted from muscular structures of the species *Conger conger*. This compound displays a powerful anti-tumoral effect in many different tumor cell-lines, with specific effects in colon cancer, ulcerative colitis, and Crohn's disease [6].

E-MHK-0103 is an atypical LP derived from the Atlantic mollusc *Mytilus galloprovincialis* cultivated on the Atlantic coast of Galicia (Spain). This bioproduct regulates hypothalamus-pituitary hormones, influences growth and development, protects against menopause-related biological decline, and modulates bone metabolism, acting as a powerful anti-osteoporotic agent [10,11].

E-SAR-94010 (Sardilipin, LipoEsar[®], LipoSea[®]) is an LP obtained from the species *Sardina pilchardus* [1]. The main chemical compounds of LipoEsar[®] are lipoproteins (60-80%) whose micelle structure probably mimics that of physiological lipoproteins involved in lipid metabolism. In preclinical studies, sardilipin has been shown to be effective in (i) reducing blood cholesterol, triglyceride, uric acid, and glucose levels, as well as liver alanine aminotransferase, and aspartate aminotransferase activity; (ii) enhancing immunological function by regulating both lymphocyte and microglia activity; (iii) inducing antioxidant effects mediated by superoxide dismutase activity; and (iv) improving cognitive function [2]. This LP shows a powerful effect in the regulation of lipid metabolism, especially by reducing total-cholesterol and LDL-cholesterol levels in cases of dyslipidemia or hypercholesterolemia, and also acting as an effective co-adjuvant of statins. E-SAR is effective in liver steatosis and in cases of primary or secondary transaminitis. It is also a strong anti-atherogenic agent, reducing the size of atheroma plaques in systemic

atherosclerosis. E-SAR has shown cognitive-enhancing properties in hypercholesterolemic patients with Alzheimer's disease. The therapeutic response of patients with dyslipidemia to sardilipin is APOE-related. The best responders are patients with the APOE-3/3>APOE-3/4>APOE-4/4 genotypes. Patients with the other APOE genotypes (2/2, 2/3, 2/4) do not show any hypolipemic response to this novel compound. In patients with dementia, the effects of sardilipin are very similar to those observed in patients with chronic dyslipidemia, suggesting that the lipid-lowering properties of sardilipin are APOE-dependent [2,7-9,12,13].

E-JUR-94013 (DefenVid[®]) is an LF derived from the fish *Trachurus trachurus*, with anti-inflammatory activity and powerful immune-enhancing properties in cases of immunodeficiency, microbial infections and/or diseases in which there is a functional compromise of the immune system [3-5]. DefenVid significantly modifies White Blood Cell (WBC) numbers in a differential fashion, decreasing neutrophils, increasing lymphocytes, monocytes, and eosinophils, and not affecting basophils. The effect of DefenVid is immunomodulatory due to the fact that in cases with high WBC numbers the general tendency is to reduce the excess of WBC, whereas in cases with low levels of WBC DefenVid tends to increase WBC, approaching normal levels. This immunomodulatory effect of DefenVid is influenced, in part, by Single-Nucleotide Polymorphism (SNP) variation associated at least with the IL1B, IL6, and TNF genes, classically involved in neuroimmune regulation and inflammatory reactions. In patients with immunodeficient phenotypes, DefenVid reduces blood cholesterol levels in over 60% of the cases, similarly to LipoEsar in dyslipidemic patients. A differential pattern of cholesterol response to DefenVid was also associated with the IL1B-T3954C, IL6-G174C, IL6R-A1510C, and TNFA-G308A variants, which are involved in inflammatory reactions associated with atherogenesis [14]. These data, together with those reported on the APOE-dependent anti-atherogenic effect of LipoEsar [9], suggest that this class of LFs might be useful to prevent arteriosclerosis and vascular risk, either peripheral or central, in the hypercholesterolemic population and in NDDs [7,8,14].

E-PodoFavalin-15999 (Atremorine[®]) is a novel biopharmaceutical compound, obtained from structural components of *Vicia faba* L. by means of non-denaturing biotechnological processes, for the prevention and treatment of Parkinson's Disease (PD) [15,16]. In vitro studies revealed that Atremorine is a powerful neuroprotectant in (i) cell cultures of human neuroblastoma SH-SY5Y cells; (ii) hippocampal slices in conditions of oxygen and glucose deprivation; and (iii) striatal slices under conditions of neurotoxicity induced by 6-OHDA. In vivo studies showed that Atremorine (i) protects against 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration; (ii) inhibits MPTP-induced microglia activation

and neurotoxicity in substantia nigra; and (iii) improves motor function in mice with MPTP-induced neurodegeneration [16].

Clinical studies have been performed in 3 groups of patients: (i) NP: drug-free patients with PD; (iii) AP: Parkinsonian patients chronically treated with L-Dopa; and (iii) MX: a heterogeneous sample of patients with Parkinsonian disorders. One hour after a single dose (5 g) of Atremorine, plasma levels of dopamine increased from 16.71 ± 14.38 pg/mL to $2,286 \pm 4,218$ pg/mL ($p < 0.001$) in NP, from $4,149 \pm 7,062$ pg/mL to $13,539 \pm 12,408$ pg/mL ($p < 0.001$) in AP, and from $860 \pm 3,445$ pg/mL to $4,583 \pm 8,084$ pg/mL ($p < 0.001$) in MX patients, with a parallel clinical improvement lasting for 3-6 h. Atremorine administration also increased the plasma levels of noradrenaline in NP and MX. Atremorine decreased prolactin levels in NP and MX, and Growth Hormone (GH) levels in NP and MX. Pharmacogenetic studies indicate that the therapeutic response induced by Atremorine in PD is associated with the pharmacogenetic profile of each patient.

LFs and Favalins are clear examples of novel bioproducts with genotype-dependent beneficial effects in complex disorders such as cardiovascular, oncological and brain diseases.

References

1. Lombardi VRM, Cacabelos R (1999) E-SAR-94010: A marine fish extract obtained by advanced biotechnological methods. *Drugs Fut* 24: 167-176.
2. Cacabelos R, Vallejo AI, Lombardi V, Fernández-Novoa L, Pichel V (2004) E-SAR-94010 (LipoEsar®): A pleiotropic lipoprotein compound with powerful anti-atheromatous and lipid lowering effects. *CNS Drug Rev* 10: 200-201.
3. Lombardi VRM, Cagiao A, Fernández-Novoa L, Álvarez XA, Corzo MD, et al. (2001) Short term food supplementation effects of a fish derived extract on the immunological status of pregnant rats and their sucking pups. *Nutrition Res* 21: 1425-1434.
4. Lombardi VR, Fernández-Novoa L, Corzo D, Zas R, Cacabelos R (2002) Enhancement in immune function and growth using E-JUR-94013 supplementation. *Methods Find Exp Clin Pharmacol* 24: 573-578.
5. Lombardi VRM, Fernández-Novoa L, Etcheverría I, Seoane S, Cacabelos R (2005) Effects of fish-derived lipoprotein extracts on activation markers, Fas expression and apoptosis in peripheral blood lymphocytes. *Int Immunopharmacol* 5: 253-262.
6. Lombardi VRM, Pereira J, Etcheverría I, Fernández-Novoa L, Seoane S, et al. (2006) Improvement of immune function by means of Conger conger extract in an in vivo model of cold stress. *Food Agric Immunol* 17: 115-127.
7. Cacabelos R (2008) Pharmacogenomics in Alzheimer's disease. *Methods Mol Biol* 448: 213-357.
8. Cacabelos R, Cacabelos P, Torrellas C, Tellado I, Carril JC (2014) Pharmacogenomics of Alzheimer's disease: novel therapeutic strategies for drug development. *Methods Mol Biol* 1175: 323-556.
9. Cacabelos R, Takeda M (2006) Pharmacogenomics, nutrigenomics and future therapeutics in Alzheimer's disease. *Drugs Fut* 31: 5-146.
10. Corzo L, Rodríguez S, Lombardi V, Alejo R, Cacabelos R (2013) Biological properties of Mineraxin. *EuroEspes J (Gen-T)* 9: 31-40.
11. Corzo L, Rodríguez S, Alejo R, Fernández-Novoa L, Aliev G, et al. (2016) E-MHK-0103 (Mineraxin c): A Novel Nutraceutical with Biological Properties in Menopausal Conditions. *Curr Drug Metab*.
12. Cacabelos R (2007) Molecular pathology and pharmacogenomics in Alzheimer's disease: Polygenic-related effects of multifactorial treatments on cognition, anxiety, and depression. *Meth Find Exp Clin Pharmacol* 29: 1-91.
13. Cacabelos R (2012) World Guide for Drug Use and Pharmacogenomics. (1st edn). EuroEspes Publishing, Corunna.
14. Cacabelos R, Carril JC, Teijido O (2016) Pharmacogenomics and epigenomics of age-related neurodegenerative disorders: Strategies for drug development. In: *Anti-Aging Drugs: From Basic Research to Clinical Practice*, Vaiserman A (ed). RSC Publishing, Royal Society of Chemistry, Cambridge.
15. Cacabelos R, Fernández-Novoa L, Alejo R, Corzo L, Alcaraz M, et al. (2016) E-PodoFavalin-15999 (Atremorine®)-induced dopamine response in Parkinson's Disease: Pharmacogenetics-related effects. *J Genomic Med Pharmacogenomics* 1: 1-26.
16. Carrera I, Fernández-Novoa L, Sampedro C, Aliev G, Cacabelos R (2016) Dopaminergic neuroprotection with atremorine in Parkinson's disease. *Cur Med Chem*.