



## Serum BDNF and NGF Modulation by Olive Polyphenols in Alcoholics during Withdrawal

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### Abstract

Many studies have suggested possible relationships between the neurotrophins brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and alcohol addiction. Previous reports demonstrated severe changes in these neurotrophins in the serum of alcohol dependent patients and during withdrawal. Alcohol dependence syndromes during consumption and/or withdrawal are also characterized by elevated oxidative stress. Polyphenols, including olive polyphenols, are natural compounds known to possess marked antioxidant properties. Thus, this study was carried out in order to verify the effects of a blend of olive polyphenols supplementation containing mostly hydroxytyrosol (50 mg/day for 15 consecutive days) in alcoholic men during withdrawal on serum BDNF and NGF. As controls a group of alcohol dependent patients received sucrose tablets as placebo. BDNF and NGF were measured by ELISA on day 1, 3, 7 and 15 of the detoxification period. Some parameters of oxidative stress were analyzed too as free oxygen radicals defense (FORD) and free oxygen radicals test (FORT). No differences in oxidative status due to polyphenols were found. However, withdrawal elicited a mild increase in BDNF over two weeks that was counteracted on day 3 by polyphenols. As for NGF no effects of polyphenols supplementation were discovered to antagonize the expected NGF serum elevation during withdrawal. In conclusion the present data may indicate that monitoring serum BDNF and/or NGF in alcoholics undergoing detoxification could contribute to characterize alcohol dependence profiles to improve recovery processes throughout also antioxidant compounds.

**Key Words:** BDNF; NGF; Olive; Polyphenol; Withdrawal; Alcoholism

### Introduction

Prolonged alcohol consumption and withdrawal following chronic or prenatal [1,2] alcohol intake causes various kinds of tissue damage; this results from increased cell death or decreased cell proliferation in several regions of the brain and other target organs of ethanol intoxication [3–6]. Several anatomical studies of the alcoholic brain show that changes in the hippocampus, extrahippocampal cortex, and basal forebrain can induce memory dysfunction [7] and learning disabilities [8]. Although the mechanisms having a role in chronic alcohol treatment-induced neurotoxic actions have not yet been clearly identified, different investigations have hypothesized that alcohol intake (i.e. acute, chronic, during gestation, paternal [1,2,9,10]) may disrupt the synthesis of neurotrophins, which are a proteins' family playing a crucial role in cognitive function, including the processes of learning and memory [11,12]. Neurotrophins are particularly important in many aspects of brain cells' development, nutrition, growth and survival [12–14]. Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are the best known neurotrophins and they were reported to be involved in the pathogenesis of alcohol dependence [14] and toxicity [16]. NGF and also BDNF have also a role in regulating stress related events [17–19]. Data show that BDNF and/or NGF secretion were significantly affected by chronic ethanol treatment suggesting that these neurotrophins might be related to ethanol-induced tissue damage [20–23]. In particular some studies have shown serum BDNF/NGF modifications in human following alcohol related diseases including potentiated BDNF/NGF values during withdrawal [24–28].

The ability of alcohol to promote oxidative stress and the role of free radicals in alcohol-induced tissue injury [29–31] clearly are important areas of research in the alcohol field, particularly because such information may be of major therapeutic significance in attempts to prevent or ameliorate alcohol's toxic effects. Polyphenols, including olive polyphenols, have been shown to possess several potential biological consequences as possible reduction of inflammation, neuroprotection, prevention of coronary artery diseases including specific research on endothelial cells via down-regulation of oxidative LDL and antioxidant properties by scavenging free radicals and up-regulating certain metal chelation reactions [32–36].

Thus, we sought to investigate whether or not olive polyphenols supplementation in alcoholic patients undergoing withdrawal could modulate serum BDNF and/or NGF measured by an immunoenzymatic assay. In this study alcohol dependent patients who entered in a university hospital center for ethanol disintoxication were administered with or without a blend of olive polyphenols (50 mg/day) containing mostly hydroxytyrosol for 15 consecutive days. Their

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**Received:** June 24, 2015; **Accepted:** July 21, 2015; **Published:** July 27, 2015

**Citation:** Ceccanti M, Carito V, Vitali M, Iannuzzi S, Tarani L, et al (2015) Serum BDNF and NGF Modulation by Olive Polyphenols in Alcoholics during Withdrawal. J Alcohol Drug Depend 3: 214. doi:10.4172/23296488.1000214

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peripheral concentrations of BDNF/NGF were assessed on day 1, 3, 7 and 15 of the experimental schedule. We also investigated pro-BDNF and pro-NGF by western blots since there is also evidence showing that the precursors to both BDNF and NGF, pro-BDNF and pro-NGF, may also play important biochemical and physiological roles [37]. Some parameters of oxidative stress were analyzed too as free oxygen radicals defense (FORD) and free oxygen radicals test (FORT).

## Methods

### Participants

Participants' description is reported in Table 1. The study included at the beginning 55 alcoholics' men but following the exclusion criteria described later 21 alcoholics were recruited and were divided in two groups. A group with 11 subjects was administered with 2 tablets/day (150 mg each, based on RedoxPhenol provided by Leadergy: <http://www.leadergy.it/>) containing a blend of polyphenols for a total of 50 mg/day of polyphenols for 15 consecutive days (see Table 2 for tablet composition) extracted by the olive pomace and containing mostly hydroxytyrosol and oleuropein [35]. As controls 10 alcoholics received sucrose tablets as placebo. Blood samples were collected at the beginning of the experimental schedule (day 1). Blood was also collected after 3, 7 and 15 days (day 3/7/15) of the experimental schedule. However, only 6 participants per group fully continued the present experimental schedule and to follow a 4-level repeated measure outcome (see methods later)

		Male
<b>Subject</b>		n=6
<b>Age</b>		47.0 ± 2.08 (ns)
<b>Educational Level - 1 Low, 4 Top</b>		2.5 ± 0.15 (ns)
<b>SES - 1 Low, 4 Top</b>		2.12 ± 0.23 (ns)
<b>Years of risk consumption</b>		21.7 ± 2.11
<b>Alcohol Preference (%)</b>	<b>Wine</b>	44.38%
	<b>Beer</b>	24.25%
	<b>Spirit</b>	29.48%
	<b>Other</b>	1.89%
<b>Previous use of psychoactive substances (%)</b>		49.15%
<b>Smoking (daily number of cigarettes)</b>		20.5 ± 2.29 (ns)

Alcoholics administered with placebo

		Male
<b>Subject</b>		n=6
<b>Age</b>		50.7 ± 2.11
<b>Educational Level - 1 Low, 4 Top</b>		2.2 ± 0.15
<b>SES - 1 Low, 4 Top</b>		2.13 ± 0.45
<b>Years of risk consumption</b>		20.3 ± 1.98
<b>Alcohol Preference (%)</b>	<b>Wine</b>	50.87%
	<b>Beer</b>	38.29%
	<b>Spirit</b>	9.82%
	<b>Other</b>	1.02%
<b>Previous use of psychoactive substances (%)</b>		44.58%
<b>Smoking (daily number of cigarettes)</b>		18.91 ± 2.34

Alcoholics administered with a blend of olive polyphenols containing mostly hydroxytyrosol (50 mg/day for 15 consecutive days)

**Table 1:** Description of male alcoholic patients administered with polyphenols and relative controls (alcoholics administered with sucrose as placebo). Data are expressed as mean ± SEM or as percentage. (nm: not measurable; ns: not significant between groups). SES = Socio-economic status. According to NIAAA for men alcohol risk consumption begins with more than 4 drinks on any single day and more than 14 drinks per week. 1 drink = 12 g of alcohol in Italy.

Phenolic Families	(in milligrams)
Hydroxytyrosol	9
Oleuropein	2
Other polyphenols as Secoiridoid acids	-
Oleocanthal - Ligstroside and derivatives	-
Other phenolic acids	14
Total polyphenols content	25
<b>Excipients and Bulking Agents</b>	
(Cellulose – Polyvinil pirrolidone – Stearic Acid – Glycerol – Silicon Dioxide – Hydroxypropyl Methylcellulose – Sodium Carboxymethyl cellulose)	75

**Table 2:** Tablet composition (100 mg each)

statistical analysis was carried out on a n=6. The alcoholics subjects were recruited in the "Centro di Riferimento Alcolologico della Regione Lazio" of Policlinico Umberto I, Sapienza University Hospital, in Rome, Italy. A trained psychologist conducted diagnostic clinical interviews by using the Structured Clinical Interview for Diagnostic and Statistical Manual (DSM-IV) Non-Patient Edition (SCID-I/NP) (First, 1997). All recruited alcoholics met the DSM IV criteria for alcohol dependence. The alcoholics also underwent two semi-structured interviews to assess lifetime alcohol consumption. The Life Drinking History-L.D.H. (Skinner and Sheu, 1982) and Time Line Follow Back –T.L.F.B. (Sobell, 1992) were used to assess alcohol consumption from the first year of regular drinking and specific amounts of alcohol consumed over the past six months respectively. Patient history of alcohol drinking behavior was based also on family history of alcohol dependence. Each patient's smoking history was also assessed. None of the subjects who were recruited showed abnormal findings in laboratory screening tests. Exclusion criteria for all participants included history of head injury, loss of consciousness, history of organic mental disorder, previous assumption of psychoactive drugs (as cocaine, opioids, amphetamine, other recreational drugs, anxiolytics, euphorants, antipsychotics, barbiturates, benzodiazepines, antidepressants, hallucinogens – data based on urine toxicology), seizure disorder or central nervous system diseases and no sign of hypertension at the time of recruitment. Blood alcohol levels were measured in all participants by using Alcoscan AL7000. All of the subjects provided written informed consent after receiving a complete description of the study. The study was approved by the university hospital ethics committee at (Sapienza Università di Roma, Italy), and all study procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983, for human experimentation.

### Free Oxygen Radicals Defense (FORD) and Free Oxygen Radicals Test (FORT)

FORD and FORT tests were carried out using two specific kits (both purchased by Callegari, Parma, Italia) following the instruction of the manufacturer. Blood serum was used both for the FORT and FORD determination. FORD test allows the determination of free oxygen radical defense. Briefly, this test uses preformed stable and colored radicals and determines the decrease in absorbance that is proportional to the blood antioxidant concentration of the sample according to Lambert Beer's law [38]. In the presence of an acidic buffer (pH = 5.2) and a suitable oxidant (FeCl<sub>3</sub>) the chromogen, which contains 4-Amino-N,N-diethylaniline sulfate forms a stable and colored radical cation photometrically detectable at 505 nm. Antioxidant compounds in the sample reduce the radical cation of the chromogen quenching the color and producing a decoloration of the solution, which is proportional to their concentration. The absorbance values obtained for the samples

are compared with a standard curve obtained using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a permeable cell derivative of vitamin E commonly employed as an antioxidant. Instead, FORT test allows the determination of free oxygen radicals (ROS), through a colorimetric assay based on the ability of transition metals, such as iron, to catalyze the breakdown of hydroperoxides (ROOH) into derivative radicals, according to Fenton's reaction. Briefly, when 20  $\mu$ L of the blood serum sample was dissolved in an acidic buffer, the hydroperoxides reacted with the transition metal ions liberated from the proteins in the acidic medium and were converted to alkoxy- (RO) and peroxy- (ROO) radicals.

The radical species produced by the reaction interact with an additive (phenylendiamine derivative (2CrNH<sub>2</sub>)) that forms a colored, fairly long-lived radical cation evaluable by spectrophotometer at 505 nm (linear kinetic-based reaction, 37 °C). The intensity of the color correlates directly to the quantity of radical compounds and to the hydroperoxide concentration and, consequently, to the oxidative status of the sample according to the Lambert Beer law (Form CR 2000; Callegari, Parma, Italy).

### BDNF and NGF determination

NGF and BDNF were measured following indications previously released (2) in the blood serum of the subjects. NGF/BDNF evaluation was carried out with ELISA kits "NGF Emax<sup>TM</sup> ImmunoAssay System number G7631" and "BDNF Emax<sup>TM</sup> ImmunoAssay System number G7611" by Promega (Madison, WI, USA) following the instructions provided by the manufacturer. The colorimetric reaction product was measured at 450 nm using a microplate reader (Dynatech MR 5000, PBI International, USA). NGF/BDNF concentrations were determined, from the regression line for the NGF/BDNF standard (ranging from 7.8 to 500 pg/ml purified NGF or BDNF) incubated under similar conditions in each assay. Under these conditions, the recovery of NGF or BDNF in our assay ranged from 80 to 90%. The NGF sensitivity of the assay was about 3 pg/g of wet tissue and cross-reactivity with other related neurotrophic factor (BDNF, neurotrophin-3 and neurotrophin-4) was less than 3%. The BDNF sensitivity of the assay was about 15 pg/ml of wet tissue and cross-reactivity with other related neurotrophic factor (NGF, neurotrophin-3 and neurotrophin-4) was less than 3%. Data are represented as pg/mg total proteins and all assays were performed in duplicate which were averaged for statistical comparison.

### pro-BDNF and pro-NGF determination

Blood serum was used for total protein concentration measured by the Bradford method, and Western blot studies as described. For WB analysis, the samples (30 $\mu$ g total protein) were dissolved in loading buffer (0.1 mol/l Tris-HCl buffer, pH 6.8, containing 0.2 mol/l DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by SDS-PAGE, and electrophoretically transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes. The membranes were incubated for 1h at room temperature with 5% non-fat dry milk dissolved in TBST (10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl and 0.1% Tween-20), washed three times for 10 min each in TBST and then incubated overnight at 4°C with primary antibodies, such as pro-NGF 1:1000 (provided by Alomone labs, Israel, catalog number: ANT-005) and pro-BDNF 1:1000 (provided by Millipore, Billerica, Massachusetts, USA, catalog number: AB5613P). Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signalling Technology, Danvers, MA, USA), or horseradish peroxidase-conjugated anti-rabbit, or anti-mouse IgG (Cell Signalling Technology) were used as the secondary antibody.

The blots were developed with ECL Chemiluminescent HRP Substrate (Millipore, Billerica, Massachusetts, USA) such as the chromophore. The public domain Image J software (<http://rsb.info.nih.gov/ij/>) was used for gel densitometry and protein quantification following the method described at <http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/>. The integrated density of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the normalizing factor.

### Statistical Analysis

ANOVAs with the polyphenols supplementation as independent variable were used to analyze BDNF, NGF, pro-BDNF and pro-NGF. All data were also analyzed with a 4-level repeated measure outcome (day 0, 3, 7 and 15 of the experimental schedule).

### Results

No differences between groups due to withdrawal or polyphenols supplementation in alcoholics were found in FORD and Fort parameters in alcoholics.

Figure 1 shows the data on BDNF and NGF ELISAs in the serum of alcoholics administered or not with polyphenols. Although in the absence of a main effect of polyphenols throughout the test on serum BDNF [ $F(1,10)=0.55$ ,  $p=0.47$  for the main effect of polyphenols] panel A shows a significant difference between groups on day 3 of the experimental schedule in BDNF levels with a marked decrease due to polyphenols [ $F(3,30)=3.99$ ,  $p=0.016$  for the interaction polyphenols  $\times$  repeated measures]. ANOVA of the repeated measures parameter also revealed a significant effect with an increase evident on day 15 of the experimental schedule [ $F(3,30)=5.93$ ,  $p=0.002$ ].

Figure 2 As for NGF (panel B) no effects of polyphenols' supplementation were detected but only an effect of the repeated measures throughout the investigation with the highest values on days 3 and 7 [ $F(3,30)=2.87$ ,  $p=0.052$  for the effect of the repeated measures].

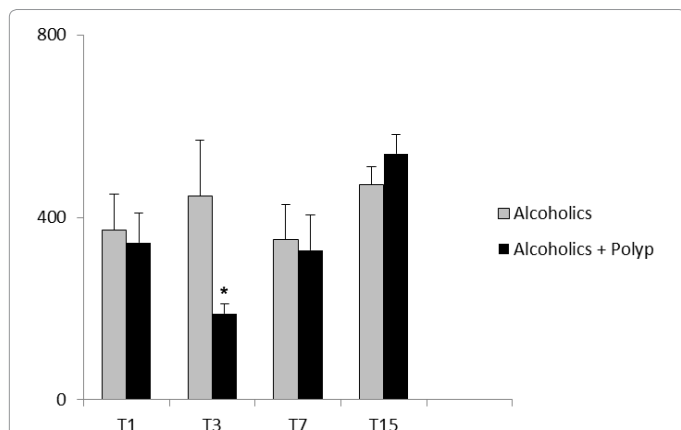
As for both pro-BDNF and pro-NGF, western blot analyses showed the presence of the pro-BDNF band at 35 kDa and of the pro-NGF band at 32 kDa. However, since both pro-BDNF/NGF expressions were highly variable between subjects, ANOVA did not detect significant differences between groups due to polyphenols supplementation or withdrawal throughout the test (data not shown).

### Discussion

This is the first study to demonstrate that administration of olive polyphenols in alcoholics undergoing withdrawal may modulate serum BDNF with a decrease. BDNF data also showed that withdrawal is going to potentiate serum BDNF in alcoholics as shown by the ANOVA of the repeated measures. As for NGF, data showed no effects of olive polyphenols but a serum NGF elevation during the first week of withdrawal. Quite interestingly, these effects on serum BDNF/NGF were not associated with changes in some parameters of oxidative stress as FORD and FORT.

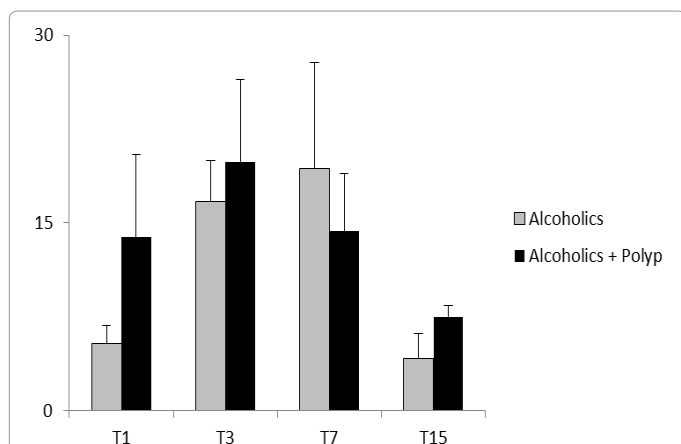
Polyphenols, including olive polyphenols, are considered to be antioxidants for their ability to scavenge free radicals and up-regulate certain metal chelation reactions [32,39,40]. However, their real ability to counteract oxidative stress in vivo appear to be dose dependent and sometime still debated [32,41]. Indeed, mouse studies showed that ip administration of a blend of olive polyphenols containing mostly hydroxytyrosol induced antioxidant effects [35] whereas ip administration of a blend of olive polyphenols containing mostly





**Figure1:** BDNF serum level.

BDNF serum levels expressed as pg/g proteins of male alcoholics with or without olive polyphenols supplementation. Blood samples were collected at the beginning of the experimental schedule (T1). Blood was also collected after 3, 7 and 15 days (T3, T7 and T15) of the experimental schedule. The vertical lines in the figure indicate pooled standard error means (SEM) derived from appropriate error mean square in the ANOVA. Asterisks indicate significant differences between groups (\* $p < 0.05$ ).



**Figure2:** NGF serum level.

NGF serum levels expressed as pg/g proteins of male alcoholics with or without olive polyphenols supplementation. Blood samples were collected at the beginning of the experimental schedule (T1). Blood was also collected after 3, 7 and 15 days (T3, T7 and T15) of the experimental schedule. The vertical lines in the figure indicate pooled standard error means (SEM) derived from appropriate error mean square in the ANOVA.

oleuropein had pro-oxidant effects [36]. Both studies evidenced also neurobiological effects due to polyphenols supplementation at brain BDNF and NGF levels. On the whole, consuming dietary polyphenols from extra virgin olive oil resulted to be associated with healthy effects in humans such as possible reduction in inflammatory processes or protection against coronary artery diseases including specific effects on endothelial cells via down-regulation of oxidative LDL (28 for review). Hence, this wide spectrum of effects could have contributed to modulate a decrease in BDNF in the serum of alcoholics during withdrawal.

As for BDNF and NGF levels per se during alcohol addiction, there is now considerable information showing that alcohol decreases the synthesis, availability, delivery and biological activity of these neurotrophins altering the capability of target neurons to respond to

these factors [9,42]. The most commonly known effects on neurotrophins of chronic alcohol addiction are disrupted nerve outgrowth, impaired neuronal survival, limited ability to promote neuronal regeneration, and alterations in the neurochemical phenotypes of selected neuronal cell lines [22,43]. Furthermore BDNF and NGF expressions are also known to be affected by ethanol exposure in fetus or by paternal alcohol consumption [2,21]. Thus, circulating BDNF and/or NGF alteration could have a critical role in chronic alcohol-induced neurotoxicity.

We found that under the present experimental conditions 2 weeks long withdrawal is going to mild potentiate serum BDNF in alcoholics as shown by the ANOVA of the repeated measures. Changes in circulating BDNF following alcohol addiction or withdrawal have been widely demonstrated in previous studies [26,27,44–47]. In particular, decreased serum BDNF concentrations was found in patients suffering from alcohol dependence which dramatically increase after acute withdrawal [26]. Another study addressing changes in serum or plasma BDNF in alcoholics revealed that higher serum BDNF levels were observed in alcohol dependent patients compared to controls [27]. However, no differences in plasma BDNF levels were observed in the same two previous groups [27]. Long-lasting withdrawal (6 months) elicits also high serum BDNF [44] as well as one week after alcohol withdrawal [48]. As for NGF our findings indicate a serum NGF potentiation during the first week of withdrawal, data in line with a previously published observation showing elevated NGF levels in human plasma during alcohol withdrawal [28]. Indeed, authors discussed that withdrawal from chronic alcohol consumption may elicit significant stress and related anxiety triggering NGF blood release as previously observed for other stressing conditions [11,17]. However, other data showed lowered NGF levels in alcohol-dependent patients [26,49,50]. In one of these study alcoholics were abstinent for 30 days or longer to ruled out alcohol withdrawal effects such as anxiety [49]. Thus it may be hypothesized that different circulating BDNF/NGF levels due to withdrawal or long-lasting abstinence or dependence or intoxication may reflect a trait marker of alcohol addiction rather than a state marker [26,28,49,51,52].

There are some implications in a human study dealing with serum BDNF/NGF in alcoholics. Firstly, it cannot be excluded the possibility that peripheral changes in BDNF/NGF in alcoholics could reflect changes in BDNF/NGF in the brain including those associated with polyphenols supplementations since i: intracerebroventricular neurotrophins injections can influence the physiology of peripheral cells [53]; ii: under certain pathophysiological conditions neurotrophins can cross the blood–brain barrier [54,55]. iii: chronic, acute or prenatal alcohol exposure is known to affect brain BDNF and/or NGF in several brain areas [2,10,21,28,56]. Secondly, alcoholics of our samples were smokers with also a long history of smoking and nicotine is known to affect the expression of both BDNF and NGF [57,58] including, then, the possibility that nicotine-use history in alcoholics may have contributed to the present data. Thirdly, as the several studies evidenced an epigenetic down regulation of NGF during alcohol withdrawal [59] or an epigenetic control of BDNF [60] and relations between BDNF Val66Met polymorphisms and alcohol-related phenotypes [60–63] it would be of interest to provide data regarding the epigenetic control of neurotrophins' signaling and synaptic plasticity to provide promising information to understand the mechanisms mediating the interaction between stress and alcoholism.

Another implication of the present investigation together with previous findings on serum/plasma BDNF/NGF is that these neurotrophins seem to not correlate with chronic alcohol consumption

and associated toxicity but could be related to both neuronal aspects and stress related outcomes of alcohol consumption and dependence. The changes in serum levels of BDNF/NGF may indicate the concomitant activation of neurotrophins' synthesis leading to neuronal remodeling triggered by alcohol withdrawal and stress related events with a neurotrophins' crucial role in the long-term maintenance of abstinence. Thus monitoring serum BDNF/NGF in alcoholics undergoing detoxification could contribute to characterize alcohol dependence profiles to improve recovery processes.

Limitations of the present investigations consist that our sample is represented by the few number of alcoholics per groups and by the fact that we studied only male alcoholics. Indeed gender differences in alcohol addiction have been reported [64] as well as gender differences are well known in the neurotrophins' physiology [65,66].

In conclusion as alcohol addiction together with alcohol withdrawal are critical points of the recovery process from alcohol dependence the present investigation may be a further step in the attempt to unravel the pathophysiological processes involved in such events. Furthermore, this study may attract and, hopefully, yield significant interests by those working on human addiction.

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**Citation:** Ceccantia M, Carito V, Vitali M, Iannuzzi S, Tarani L, et al (2015) Serum BDNF and NGF Modulation by Olive Polyphenols in Alcoholics during Withdrawal. *J Alcohol Drug Depend* 3: 214. doi:[10.4172/23296488.1000214](https://doi.org/10.4172/23296488.1000214)