

# Cyanocobalamin Absorption Abnormality in Alcoholics is Improved by Oral Supplementation with a Fermented Papaya-derived Antioxidant

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## ABSTRACT

**Background/Aims:** Thirty alcoholic patients and 24 teetotaler dyspeptic patients were considered and underwent baseline blood chemical evaluation and the Schilling test.

**Methodology:** During gastroscopy, biopsy samples were taken to assay: routine histology, malonyldialdehyde, vitamin E and glutathione concentration and for testing vitamin B12 - Intrinsic Factor binding. Examinations were repeated after 1-week supplementation with Immun'Age.

**Results:** Plasma malonyldialdehyde level and lipid hydroperoxides concentration as well as either malonyldialdehyde and xanthine oxidase concentration in the gastric mucosa in alcoholics were significantly higher than in controls and despite unchanged alcohol consumption, significantly decreased after Immun'Age supplementation.

Gastric mucosal glutathione was markedly depressed in alcoholics and partly recovered after Immun'Age supplementation. Although the alcoholics

showed a normal intrinsic factor secretion in the gastric juice, they exhibited a markedly depressed intrinsic factor-cobalamin binding on the "ex vivo" study. Moreover, nearly 23% of them had an abnormal Schilling test. Both these impairments reverted to normal after Immun'Age supplementation.

**Conclusions:** It can be postulated that the antioxidative action played by Immun'Age, possibly due to its availability substrates for glutathione synthesis as well as to its effects on local oxidative burst from neutrophil. is able to recover a normal cobalamin absorption.

## KEY WORDS:

Cyanocobalamin absorption, Intrinsic factor binding; Free-radical damage; Immun'Age

## ABBREVIATIONS:

Hepatitis B Virus (HBV); Antibodies to Hepatitis B Virus (anti-HCV), High Performance Liquid Chromatography (HPLC), Superoxide Dismutase (SOD), Dual-isotope Technique (DIST) Intrinsic Factor (IF); Thiobarbituric Reactive Substances (TBARS)

## INTRODUCTION

Cobalamin malabsorption is likely to be a largely underrated clinical entity and its spectrum covers a wide number of mild-to-undiagnosed cases. It has been shown in humans that the administration of alcohol to healthy volunteers brings about a decrease in vitamin B12 absorption, as measured by the Schilling test (1). Accordingly, there are albeit scanty reports that alcoholics may have low serum cobalamin levels, which in some cases may revert to normal after prolonged abstinence (2).

Alcoholics' gastritis has been generally indicated as a background cause of this phenomenon although such clinical entity has been questioned for a long time. Shaw et al (3) have suggested by experimental work that ethanol-induced reactive oxygen species may hamper gastric intrinsic factor binding of B12. We have very recently shown that a novel oral antioxidant, obtained from yeast fermented mixture of medicinal plants (*Carica papaya*, *pennisetum purpureum*, *Sechium edule*) is able to markedly decrease the oxidative stress in alcoholics taking place during the early phase of abstinence (4). The aim of the present study was thus to gain further insight into the presumptive free radical-mediated mechanism of

cyanocobalamin malabsorption in a clinical setting of alcoholics while testing at the same time the potential protective effect of Immun'Age supplementation.

## METHODOLOGY

Patients with long-standing elevated alcoholic consumption were considered (>150g ethanol/day for at least 3 years). All patients underwent a careful personal interview to establish ethanol consumption and drinking behavior. A detailed diet-history method was carried out and nutrient intakes were calculated by using a standardized food-composit ion table (Table 1). Drinking habit was mostly wine at meal times and between meals. There was no concomitant or prior regular consumption of medications or drugs at the entry into the study. In particular, 9 patients admitted an occasional use of antacids or a short course of H<sub>2</sub> blockers or, less frequently, proton-pump inhibitors for the past 18 months

Nearly 25% of all alcoholics who were initially screened, had been admitted to hospital during the previous 12 months. On these occasions, some of them had received parenteral thiamine administration but none had been given cyanocobalamin supplementation under any formulation. All of them had abnor -

**TABLE 1 Dietary Intake in Alcoholic Population**

Total energy (kcal)	2816±423
Non-alcohol energy (kcal)	1879±253
Protein (%)	14.4±2.7
Carbohydrate (%) *	58.7±8.7
Fat (%)	26.5±5.3
Vitamin A (µg) **	1512±789
Vitamin E (mg)	8.2±1.9
Vitamin C (mg)	82.8±23.7
* percentage of non-alcohol energy	
** µg equivalent retinol = retinol (g) + carotene (µg)/6	

**TABLE 2 Chemical Analysis of Immun'Age (100G)**

Available carbohydrate:	90.7g
Moisture	8.9g
Protein	0.3g
Fat	none
Ash	0.1g
Folk acid	2µg
<b>Amino acids</b>	
Arginine	16mg
Lysine	6mg
Histidine	5mg
Phenylalanine	11mg
Tyrosine	9mg
Leucine	18mg
Isoleucine	9mg
Methionine	5mg
Valine	13mg
Alanine	12mg
Glycine	11mg
Proline	8mg
Glutamic acid	37mg
Serine	11mg
Threonine	8mg
Aspartic acid	27mg
Tryptophan	2mg

Carotene, cobalamin, thiamin, Riboflavin, total ascorbic acid, tocopherols, selenium: not detected  
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mal liver function tests and with an over 4-fold increase of gamma-glutamyl-transpeptidase value. Two patients were positive for antibodies to hepatitis B virus (anti-HCV) and none had positive markers of ongoing hepatitis B virus (HBV) infection.

There was no evidence of pancreatic insufficiency or intestinal bacterial overgrowth. As a control group, teetotaler subjects undergoing gastrointestinal investigations for non-ulcer-like dyspepsia and devoid of peptic ulcer or any other relevant gastrointestinal disease were considered. Both patients and control subjects had not used vitamin supplements. A positive urease test (FUD test, Medimar, Milano), whose high sensitivity and specificity had already been confirmed in our routine practice, was used as an exclusion criteria for all subjects involved in the study. Therefore, 30 patients (25 males and 5 females, mean age: 46 years; range: 42-53 years) and 24 control subjects were finally selected for the study.

In the alcoholic group, 21 patients were smokers and 8 of them heavy smokers (over 40 cigarettes/day) while 11 control subjects were smokers and 2 of them were heavy smokers (not statistically significant difference).

### Study Design

Patients were divided in 2 groups comparable as for age, gender, drinking and smoking habits. After overnight fasting, all patients underwent baseline blood chemical evaluation as described below. Urine samples were collected for the Schilling test.

During upper gastrointestinal endoscopy, biopsy samples of adequate size were taken each from the antrum and the gastric body. These serve the purpose of assessing routine histology, malonyldialdehyde (MDA) level, vitamin E and glutathione concentration and for assay vitamin B12 binding by intrinsic factor (IF). Further, gastric juice samples were collected and stored for assaying intrinsic factor and xanthine oxidase concentration. Endoscopic and biochemical assessments were repeated after 1 week of supplementation with Immun'Age (Composition showed in **Table 2**, Osato Research Foundation, Gifu, Japan) 9g/nocte. During such period patients continued in their dietary habits and, in particular, with unchanged alcohol consumption, as ascertained later on.

### Biochemical Methodology

**Plasma biochemical measurement:** Anti-oxidant status: Blood samples were taken using EDTA and anticlotting agent and immediately refrigerated in ice. The plasma was separated by centrifugation at 4°C. Plasma malonyldialdehyde as assayed by the method of Yagi (5) was modified by adding 0.01% butylated-hydroxytoluene to the coloring solution to avoid the generation of thiobarbituric reactive substances (TBARS) reactive molecules during the procedure. The system was heated at 100°C for 45min. After cooling, the chromophore was extracted with 2 mL of N-butanol by vigorous shaking and dried under constant nitrogen flux. The final powder was resuspended in 100µL of chromatographic solvent A and added to a high performance liquid chromatography (HPLC) system (solvent A: 10mmol hexate sulfonic acid and H3PO4 adjusted to pH 3; solvent B; methanol). Tetra-ethoxy-propane served as a standard source of malonyldialdehyde which is a TBARS. All samples were processed in duplicate. Glutathione peroxidase and superoxide dismutase (SOD) activities of plasma were determined in a centrifugal analyser (Hoffman La Roche, Switzerland). Plasma lipid hydroperoxides, mostly phospholipid hydroperoxides, were assessed by hemoglobin catalyzed oxidation of 10-N'-methylcarmoyl-3, 7-diemthylamino-10-H-phenothiazine after treatment with phospholipase D. Cumene hydroperoxide was used as standard. Plasma phospholipid was evaluated by a commercial available enzymatic kit (Boehringer Biochemia, Mannheim, Germany).

**Gastric mucosal biochemical parameter:** Malonyldialdehyde concentration in the gastric

mucosa was determined as outlined above. Xanthine oxidase activity was assessed by applying to tissue the methodology cited below for gastric juice. For alpha-tocopherol determination pre-weighed biopsies were removed from the freezer and kept in ice to then be quickly blotted and weighed. Each biopsy sample was placed in a 5-mL tube with traces of butylated hydroxytoluene crystals and 20 $\mu$ L of a freshly made collagenase solution (20g/L containing 5mmol CaCl<sub>2</sub>/L). The tubes were incubated in a water bath at 45°C for 15min. On removal from the bath, 20 $\mu$ L of a freshly made pronase E solution (30g/L in 0.02mL phosphate buffer/L, pH 7.4) was added to each tube which was placed in ice. Each softened biopsy mixed with butylated hydroxytoluene crystals and enzymes was transferred to a glass microhomogenizer for 90s. The homogenate was then returned to its original tube. The homogenizer was washed twice by adding either 40 $\mu$ L of 5mmol CaCl<sub>2</sub>/L and 40 $\mu$ L of water. The tubes were finally incubated in a water bath at 45°C for 15min. Soon afterwards, a quick extraction of a-tocopherol was carried out as follows. A freshly prepared solution (0.12mL) of 95% ethanol containing 800mg ascorbic acid/L, 0.55g sodium dodecyl sulphate/L and 125mg tocopherol acetate/L was added to each tube. The solution was immediately vortex-mixed for 4s. Tubes were then placed in a multi-vortexer for 2min, centrifuged at 200 x g for 3 min at 20°C and the upper hexane layer was transferred to a clean 5-mL tube. The residue was extracted a second time with a further 0.7mL hexane and shaken for 30s. The tubes were centrifuged and the supernatant added to the first hexane extract. The solvent was blown off by a stream of nitrogen at 40°C. the residue was reconstituted in 60 $\mu$ L ethanol and transferred to 0.3-mL autosampler vials which were stored at -70°C: the following day the specimens were injected by using the same mobile phase. To assay glutathione concentration, tissue samples were homogenized in EDTA 0.02M at 4°C in the presence of 10 $\mu$ L of 50mM monobromobimane in acetonitrile and 20 $\mu$ L of 50mM *N*-ethylmorpholine at pH 8.4 for the derivatization of the sulphhydryl groups. After 15min reaction in the dark, protein precipitation was obtained by 10% sulphosalicylic acid and final removal by centrifugation at 13.800rpm for 5min. The supernatant was filtered through a 0.45- $\mu$ m Millipore and injected into a HPLC system. Excitation and emission wavelength were 370nm and 485nm, respectively. As derivatization standards, 10-100nmol of glutathione were injected into HPLC system prior to each sample processing.

**Assay of intrinsic factor and xanthine oxidase in the gastric juice:** For the intrinsic factor determination, gastric juice aliquots were depepsinized by titration to pH 11 using 10N sodium hydroxide, allowing the mixture to stand over ice for 15min and then titrating to pH7 with glacial acetic acid before storage at -20°C. Intrinsic factor concentration in gastric juice was determined by applying slight modifications to the methods described by Begley and al (61). Briefly, the standard curve was

prepared by adding known amounts of IF (Sigma Chemical Company, USA) 0-10 units (made up to 100 $\mu$ L with distilled water), to 0.01N Tris HCl buffer (0.5mL, pH 7.4), Cobinamide, 0.25 $\mu$ L was added and the tubes incubated for 30min at 37°C. This was followed by 20 $\mu$ L of <sup>57</sup>Co-labeled cyanocobalamin (Amersham Corporation, Illinois, USA) per tube and the mixture incubated for a further 30min at 37°C. Free (<sup>57</sup>Co) cyanocobalamin was separated from IF-bound (<sup>57</sup>Co) cyanocobalamin by the addition of 1mL/tube of albumin-coated charcoal. The standard curve was prepared by plotting increasing concentrations of IF against B/(B+F), where B is the radioactivity in the supernatant (IF-bound) and F the radioactivity in the charcoal. IF in gastric juice was assayed using 100 $\mu$ L of gastric juice treated as above and the concentrations determined from the standard curve. For xanthine oxidase determination, gastric juice samples were checked for pH value by a digital pH meter and centrifuged at 300rpm for 5min to obtain a clear supernatant. Supernatants were extracted by using a 3:5v/v chloroform-ethanol mixture and then centrifuged at 10000rpm for 15min. The clear upper layer thus obtained was used for xanthine oxidase activity determination, as described by Prajda et al (7). Gastric juice pH was checked by a pH meter as well.

**Schilling test:** Urine samples were used for the established dual-isotope technique (DIST), following strict methodological quality control as advised by a very recent validation study (8). Mean values were expressed as: Free <sup>57</sup>Co-Vitamin B12 excretion (%) and IF-Bound <sup>57</sup>Co-Vitamin B12 excretion (%).

**Assay of vitamin B12 binding by IF:** Pooled gastric mucosal biopsy samples from the body were immediately placed in Tris HCl, pH 10.0, homogenized in 7.5w/v Tris buffer and centrifuged at 14.000rpm for 20min at 4°C. Supernatants were diluted with saline 1:300 for IF/vitamin B12 binding assay. This was assayed following incubation of concentrated 0.5U/mL IF with reaction mixture and the solutions were diluted to 0.05 units/mL of IF for assay. For gastric homogenate incubation, a dilution of gastric samples of 1:7.5w/v was used for incubations and a final dilution of 1:300 of gastric homogenates were used for assay. Dilutes IF of gastric homogenates were added to 50pg of cyanocobalamin containing 0.025 $\mu$ Ci of <sup>57</sup>Co-cyanocobalamin. The number of counts remaining in solution after precipitation with hemoglobin-coated charcoal were determined. The specificity of the gastric homogenate IF for B12 was ascertained by incubating anti-IF antibodies stored from the serum of patients with pernicious anemia and using normal serum as control.

**Statistical analysis:** Data were analyzed using the non-parametric Mann-Whitney U test and correlations were tested using Kendall-Tau test for non-parametric data.

## RESULTS

### Serum Biochemical Assays

Routine blood chemistry did not change during the

**TABLE 3 Blood Antioxidant Status In Alcoholics: Effect of Immun'Age Supplementation**

	Non- alcoholics	Alcoholics	
		Placebo	Immun'Age
MDA ( $\mu\text{mol/L}$ )	0.27 $\pm$ 0.13	0.93 $\pm$ 0.23*	0.43 $\pm$ 0.36**
L-HPX (nmol/L)	2.3 $\pm$ 0.5	9.9 $\pm$ 0.8*	3.7 $\pm$ 1.2**
GPX(U/L)	631.2 $\pm$ 99.8	706.5 $\pm$ 112.6*	698.7 $\pm$ 128.9
SOD (U/L)	26.4 $\pm$ 7.7	33.6 $\pm$ 3.7*	30.8 $\pm$ 5.3

MDA: malonildialdehyde; L-HPX hydroperoxides; (GPX glutathione peroxidase; SOD: superoxidase dismutase

\*P<0.01 vs. non-alcoholics; \*\*P<0.05 vs. placebo group

**TABLE 4 Gastric Biochemical Parameters in Alcoholics: Effect of Immun'Age Supplementation**

	Non-alcoholics	Alcoholics	
		Placebo	Immun'Age
Malonildialdehyde (nmol/mg protein)	0.41 $\pm$ 0.03	0.97 $\pm$ 0.15*	0.55 $\pm$ 0.06**
Xanthine oxidase (mIU/mg protein)	1.12 $\pm$ 0.4	2.59 $\pm$ 0.8*	1.73 $\pm$ 1.1**
Glutathione (nmol/g wet tissue)	337.2 $\pm$ 118.4	161.3 $\pm$ 69.9*	243.5 $\pm$ 112.5**
a-tocopherol (ng/mg protein)	30.2 $\pm$ 7.4	27.7 $\pm$ 6.5	27.9 $\pm$ 8.7

\*P<0.001 vs. control; \*\*P<0.01 vs. placebo group

study period, irrespective of the treatment employed. As expected, plasma MDA level and lipid hydroperoxides concentration in chronic alcohol abusers were significantly higher than in controls (P<0.001) (Table 3). However, despite unchanged alcohol consumption, 1-week supplementation with Immun'Age brought about a significant decrease of both parameters (P<0.05), although they did not entirely revert to mean control value. Either glutathione peroxide and superoxide dismutase were significantly higher than in non-alcoholic patients (P<0.05) and remained unchanged after Immun'Age supplementation.

#### Gastric Mucosal Histology and Biochemical Assays

Routine histology showed mild-to-moderate degree of superficial chronic gastritis without *Helicobacter pylori* infection. No case of gastric atrophy was recorded. After 1-week supplementation with either placebo or Immun'Age, non-significant change occurred. Either malonyldialdehyde and xanthine oxidase concentration in the gastric mucosa in alcoholics were significantly higher than in 11011-alcoholics (P<0.001) (Table 4). After 1 week of Immun'Age supplementation such parameters significantly decreased within normal level (P<0.05). Gastric mucosal glutathione was markedly depressed in alcoholics (P<0.001) and partly recovered after Immun'Age supplementation (P<0.05 vs. placebo). On the contrary, vitamin E level in alcoholics was comparable to that of the teetotaler group and remained unaltered whatever the treatment employed.

#### Intrinsic Factor and Xanthine Oxidase Concentration in the Gastric Juice

As shown in Figure 1, the concentration of intrinsic factor in the gastric juice was within normal limits either in controls and in alcoholics and was not affected by Immun'Age treatment. Xanthine oxidase activity in gastric juice of alcoholics was significantly higher compared with the control group (P<0.01). Immun'Age treatment seemed to bring about a trend decrease of xanthine oxidase concentration but this did not reach a statistical significance. Gastric pH was within normal limit in controls and alcoholic patients either at the entry into the study and at the end.

#### Dual-isotope Schilling Test

Results are presented in Figure 2. At the entry, as compared to controls whose single values fell within normal range, 7 alcoholic patients had very low free <sup>58</sup>Co-vitamin B12 excretion percentage (below 10%) and abnormally high bound/free ratio (about 2) although this did not determine a statistically significant difference in the group as a whole. However, after 1 week of Immun'Age supplementation each single alcoholic patient showed normal values. In particular, when using a cut-off level of 10% and thus taking separately such a subgroup of alcoholics, Immun'Age supplementation enabled a statistically significant improvement (P<0.05) with values comparable to controls and with normalization of the bound/free ratio.

#### Vitamin B12 Binding by IF

As compared to control, gastric mucosa homogenates obtained from alcoholics showed a significantly reduced intrinsic factor binding of vitamin B12 (P<0.001) (Figure 3). Immun'Age supplementation for 1 week reverted to normal this abnormality (P<0.01 vs. placebo).

#### DISCUSSION

Cobalamin is a water-soluble vitamin that cannot be synthesized by mammalian species. Therefore, man is entirely dependent, on dietary sources of cobalamin whose average daily intake is theoretically 2-4-fold the physiological requirement. Its absorption relies, upon an orderly and multifaceted sequence of events which can be briefly summarized in a gastric stage, intestinal transport and uptake of intrinsic factor-cobalamin complex by ileal mucosa and cobalamin transcytosis. Binding to intrinsic factor which is present in most gastric cell types in the human stomach and whose secretion is considerably greater than the human daily requirement is the crucial event in cobalamin absorption. Shaw et al (3) has shown on experimental ground that such binding is markedly inhibited by a free radicals-generating system such as a medium of acetaldehyde and xanthine oxidase, thus providing a stimulating background for clinical studies in alcoholism. Indeed, more recently it has been suggested that the increased free radicals production observed in the presence of ethanol could be related to

the oxidation through xanthine oxidase of locally produced acetaldehyde (9). However, to the best of our knowledge, the issue of possible interference of ethanol consumption and following free radicals generation in cobalamin absorption has not been further addressed. Indeed, despite some conflicting reports, the involvement of oxygen radicals in ethanol-induced gastric injury has been extensively confirmed in "in vitro" as well as "in vivo" studies (10,11). Accordingly, in order to study a possible therapeutic intervention with antioxidants on a presumptive ethanol-induced cobalamin absorption abnormality, in the present investigation we used Immun'Age which is a novel food supplement prepared by yeast fermentation of medicinal plants and is heat- and acid-resistant (12). Although it is devoid of detectable amounts of antioxidative vitamins or enzymes (Table 2), this product has been demonstrated to scavenge hydroxyl radicals in cell-free systems (13,14) and, more recently such properties have been confirmed in clinical studies as well (4,15,16). Despite unchanged drinking habits, 1-week Immun'Age supplementation brought about a significant decrease of free radicals-related parameters either in the blood (MDA, xanthine oxidase, glutathione). The mucosa of the stomach and of the upper gastrointestinal tract is particularly rich in xanthine oxidase, the rate-limiting enzyme of purine catabolism, which generates superoxide anion as a by-product. Further, this enzyme might show an enhanced activity at the upper-gastrointestinal mucosa location under a number of local injuries with a following burst of local free radicals generation. Indeed, there is experimental evidence showing a protecting role of xanthine oxidase inhibitors on ethanol-induced gastric mucosa damage (11). When investigating in our study any possible ethanol-related cobalamin absorption abnormality, we preferred not to base it on "on spot" plasma cobalamin evaluations. Indeed, the unreliability of such determination has been critically pointed out in up-to-date reviews (17) and very recently low levels have been observed in a high percentage of non-metabolically cobalamin-deficient individuals (18). Further, we have purposely ruled out subjects or patients with *Helicobacter pylori* infection, due to its recently shown interference with food-cobalamin absorption (19). Interestingly, although the alcoholics showed a normal intrinsic factor secretion in the gastric juice, they exhibited a markedly depressed intrinsic factor-cobalamin binding in the "ex vivo" study. Moreover, nearly 23% of them had an abnormal Schilling test which is considerably higher than the 5% incidence of low serum vitamin B12 levels in alcoholics, as reported in the literature (2). Both these impairments reverted to normal after Immun'Age supplementation. Following the experimental study of Shaw et al (3), one might postulate that the antioxidative action played by Immun'Age, with significant decrease of gastric mucosal concentration of malonyldialdehyde and xanthine oxidase activity together with a partial restoration of glutathione, has enabled a recovery of normal cobalamin absorption.

## ANALYSIS OF GASTRIC JUICE

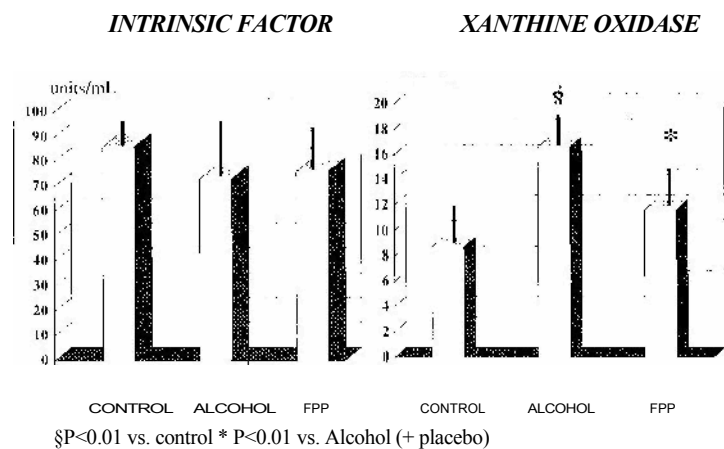


FIGURE 1 Analysis of gastric juice.

## DUAL ISOTOPE SCHILLING TEST

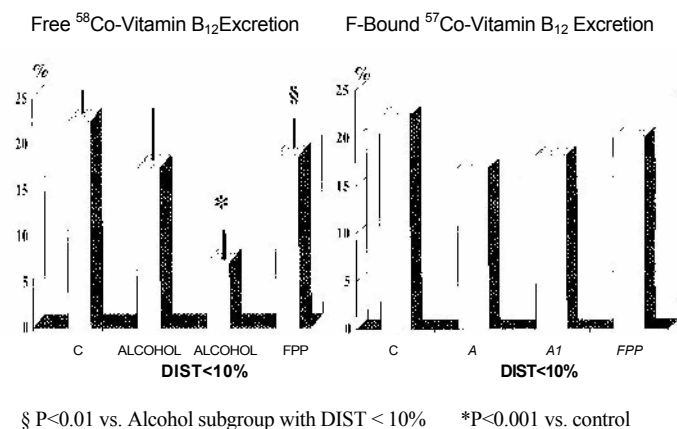


FIGURE 2 Dual-isotope schilling test

## % inhibition

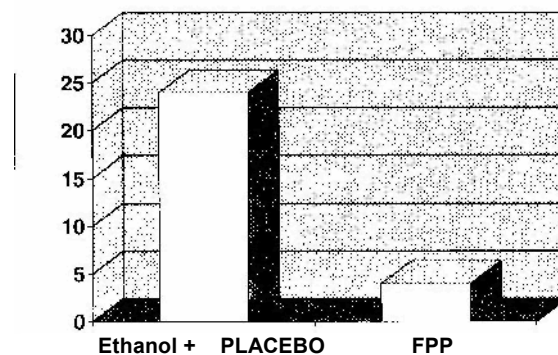


FIGURE 3 Vitamin B12 binding by IF, in vitro incubation of IF with <sup>57</sup>Co-labelled cyanocobalamin.

Our alcoholic population showed higher levels of xanthine oxidase concentration also in the gastric juice and Immun'Age supplementation caused only a not-significant trend decrease. Nonetheless, it has to be considered that the mechanism of xanthine oxidase