

**Bio-Forum Foundation**

Clinical Study Protocol No: FB-F01

Protocol Date: February 12, 2018

**Protocol ID “TiroNod”,**

**version FB-F01**

**Study ID number NCT03535974, titled:**

**“Efficacy of a Spirulina Supplement for Amelioration of benign thYroid nodules (ESSAY)”**

**The initial version of Protocol FB-F01 (in Romanian) written on February 12, 2018**

**first approved by the Ethics Committee of The Bio-Forum Foundation on March 03, 2018**

**English version (English translation) on February 24, 2020**

**Principal Investigator: Prof Dr Corin Badiu, Co-Principal Investigator: Dr Felician Stancioiu**

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# Protocol Cover Page

**Protocol Title:** Efficacy of a Spirulina Supplement for Amelioration of benign thYroid nodules (ESSAY)

**Protocol Number:** Fundatia Bio-Forum 01 (FB-F01)

**Protocol Date:** 12 February 2018

**Study Phase:** I-II

**Principal Investigator:** Prof Corin Badiu, MD, Felician Stancioiu, MD

**Research Team:** Prof Corin Badiu, MD, Daniel Mihai, MD, Felician Stancioiu, MD,

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**SPONSOR:**  
***Bio-Forum Foundation***

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**PROTOCOL SYNOPSIS**

<b>TITLE</b>	<b>Efficacy of a Spirulina Supplement for Amelioration of benign thYroid nodules (ESSAY)</b>		
<b>Investigational site:</b>	Outpatient Office, Prof Corin Badiu, National Institute of Endocrinology CI Parhon, Bucharest, Romania		
<b>Investigators:</b>	Prof Corin Badiu, Dr Felician Stancioiu, Dr Daniel Mihai		
<b>Sponsor:</b>	Bio-Forum Foundation, Bucharest, Romania		
<b>Representatives :</b>	Dr Felician Stancioiu		
<b>Study number:</b>	FB-F01		
<b>Final Protocol:</b>	February 2018	<b>Clean File:</b>	February 2018
<b>Ethics Approval:</b>	March 2018	<b>Statistical analysis:</b>	
<b>Clinical Phase:</b>	<b>I-II</b>	<b>Study Report:</b>	
<b>OBJECTIVES:</b>	To establish the safety and efficacy of a supplement containing spirulina, curcuma and boswellia in decreasing the size of benign thyroid nodules		
<b>STUDY DESIGN:</b>	double-blind, placebo-controlled, crossover clinical trial. The patient population will include approximately 30 consecutive patients initially randomized to placebo (P) or active ingredients (AI)		
<b>SUBJECTS:</b>	<b>Inclusion criteria</b> <ul style="list-style-type: none"> <li>- adult patients over 18 years of age</li> <li>- agreement to participate in the study by signing the Informed Consent Form</li> <li>- euthyroid status (normal TSH and fT4 values), no active dysfunctional thyroid disease</li> <li>- benign thyroid nodule confirmed by FNAB (Bethesda 2)</li> <li>- no previous thyroid surgery, thyroid ablation treatment, substitution treatment with thyroid hormones</li> <li>- no steroid or beta-blocker treatment</li> <li>- presence of one or more thyroid nodules documented by ultrasound</li> </ul>		

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	<p><b>Exclusion criteria</b></p> <ul style="list-style-type: none"><li>• - pediatric patients (less than 18 years of age)</li><li>• - diagnosis or suspicion of malignancy, including thyroid</li><li>• - autoimmune disease, including Hashimoto</li><li>• - Wilson disease</li><li>• - hypo- or hyper-thyroidism</li><li>• - contraception with intrauterine device</li><li>• - acute infection</li><li>• - known allergy to spirulina, curcuma or boswellia</li></ul>
<b>TREATMENT TO BE EVALUATED</b>	<p><b>Active Treatment AI:</b> Spirulina 400 mg, Curcumin 50 mg, Boswellia 50 mg; 4 capsules/day</p> <p><b>Placebo Treatment P:</b> an inert substance</p>
<b>DURATION OF STUDY</b>	6 months total, 3 months per patient
<b>ENDPOINTS:</b>	<p><b><u>EFFICACY</u></b></p> <ol style="list-style-type: none"><li>1. Reduction in the area of benign thyroid nodules</li><li>2. Examination by endocrinologist</li></ol> <p><b><u>SAFETY</u></b></p> <ol style="list-style-type: none"><li>1. Adverse events, especially allergy, nausea</li><li>2. Modification of weight, digestion.</li><li>3. Modification of laboratory tests measured from venous blood: levels of TSH, fT4, Cu</li></ol>
<b>SAMPLE SIZE:</b>	approximately 30 patients
<b>STATISTICAL ANALYSIS:</b>	Summary statistics will describe the variables. Significance testing will be done using T tests for two groups, by comparing the means and standard deviation, also Pearson correlation, and Wald Z test for proportions.. ( $\alpha = 0,05$ )

## **Introduction**

Benign thyroid nodules are among the most common endocrine disorder. Current progress in imaging and pathology stratified the risk, but apart from surgery, current treatment options are limited. Here we propose a first evaluation of a noninvasive treatment for benign thyroid nodules via an interventional, crossover, placebo controlled clinical study.

With a lifetime occurrence risk in general population estimated at 5-10% (1), thyroid nodules are a frequent pathology with a known gender bias, their prevalence being higher in women. Furthermore, along the lines of technological development with higher availability of high resolution ultrasound and other imaging modalities (ex. incidental findings on carotid or heart ultrasound, or routine scans), and also the increased focus on preventive medicine and improved access to healthcare resources, their prevalence seems to be higher than estimated, and a recent study revealed thyroid nodules in about 19-68% of randomly selected individuals (1).

Considering the treatment, besides surgery, several noninvasive procedures developed, like radiofrequency ablation, alcohol injection, high intensity focused ultrasound, or laser ablation; a minimally invasive procedure was recently shown to be a safe and efficacious treatment for benign thyroid nodules: ablation with monopolar radiofrequency (2).

This last treatment is performed with a 16- or 18- Gauge internally cooled radiofrequency electrode after local anesthesia, can treat autonomously functional nodules (3), has better results for smaller and cystic nodules, and post-procedural complications in only 17% of patients (2). Other studies have shown various results with mean decreases of 40-75% at 6 months (4,5) and minimal complications in treating benign thyroid nodules with radiofrequency ablation (5,6), being noted that key success factors are technical skill and patient selection (7).

Even though the vast majority (around 95%) of thyroid nodules are benign (1,8), and the 5% remnant thyroid malignancies are among the most successfully treated endocrine cancers (2,9), a medical noninvasive treatment which decreases the thyroid nodule dimensions would be greatly beneficial.

## **Patients**

### **Patients and methods:**

To evaluate the efficacy and safety of a supplement containing spirulina, curcuma and boswellia in euthyroid patients with benign thyroid nodules, we will perform a 3 month, double-blind, placebo-controlled study which aims to enroll approximately 30 patients. Patients with benign single thyroid nodules of less than 5 cm are evaluated in a prospective interventional placebo-controlled cross-over trial, across 12 weeks (3 visits at six weeks intervals). At each visit, the target thyroid nodule was recorded in two dimensions. In addition, plasma levels of thyroid stimulating hormone (TSH), free thyroxine (fT4) and copper (Cu) were measured.

Participants in this clinical study are consecutive patients presenting for initial evaluation or follow-up of thyroid nodules in the outpatient department of the National Institute of Endocrinology in Bucharest, Romania during May-September 2018 and fulfill the inclusion and exclusion criteria presented below. After being informed about the objective of the study, including what is needed from each participant in terms of blood samples, ultrasound evaluations, supplement administration with possible risks and benefits, the patients who agreed to the terms signed the Informed Consent Form and were enrolled in the clinical study.

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### Inclusion criteria

- adult patients over 18 years of age
- agreement to participate in the study by signing the Informed Consent Form
- euthyroid status (normal TSH and fT4 values), no active dysfunctional thyroid disease
- benign thyroid nodule confirmed by FNAB (Bethesda 2)
- no previous thyroid surgery, thyroid ablation treatment, substitution treatment with thyroid hormones
- no steroid or beta-blocker treatment
- presence of one or more thyroid nodules documented by ultrasound

### Exclusion criteria

- pediatric patients (less than 18 years of age)
- diagnosis or suspicion of malignancy, including thyroid
- autoimmune disease, including Hashimoto
- Wilson disease
- hypo- or hyper-thyroidism
- contraception with intrauterine device
- acute infection
- known allergy to spirulina, curcuma or boswellia

### **Study design**

For assessing the efficacy of the spirulina-based supplement in decreasing the size of thyroid nodules we are performing a double-blind, placebo-controlled, crossover clinical trial. The patient population will include approximately 30 consecutive cases randomized to placebo (P) or active ingredients (AI).

There are three visits for each patient, noted

V1 – initial visit at enrollment;

V2 – second (intermediary) visit, after six weeks of administration of placebo (P) or active ingredients (AI), and

V3 – final visit, after another 6 weeks of administration of P or AI.

At each visit each patient is tested for plasma TSH, fT4 and Cu levels, by an independent laboratory and a thyroid ultrasound is performed by endocrinologist (DM) using the same ultrasound machine at all study visits; the nodule images and dimensions are printed for comparison.

At V1, patients are randomly selected to receive each either AI or P, so that about half patients receive P and the other half will receive AI, and after 6 weeks, at V2, the patients previously on P will receive AI, and the patients who first receive AI receive P. At the discretion of the study investigator a few more patients will initially receive AI than P.

Consequently, some patients receive the sequence P-AI, other patients receive sequence AI-P, and yet others will receive the sequence AI-AI.

The study is registered on clinicaltrials.gov site with Protocol ID “TiroNod” and ID number NCT03535974, and titled: Efficacy of a Spirulina Supplement for Amelioration of benign thYroid nodules (ESSAY).

### **Outcomes**

The primary outcome of the study is a change in size of the thyroid nodules and this is usually evaluated by comparing the biggest diameter of the nodules between measurements. However we have

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observed that there are patients in which the largest diameter decreases while the smallest diameter increases, and this variability may bring significant bias in comparisons; therefore for more consistent results and comparisons we will calculate the product of the largest (D) and smallest (d) diameters of the nodules in the same incident angle and compare the resulting area of the nodules ( $S = D \times d$ ) measured at visits V1, 2, 3.

As secondary outcome we will measure the serum values of TSH, fT4 and copper (Cu). The first two are considered standard evaluations of the thyroid function, while the latter was chosen for multiple reasons: i. spirulina is a significant source of copper alongside with other minerals and vitamins; ii. most serum copper is bound and transported by ceruloplasmin (10), and iii. while there is an excellent correlation between serum copper and ceruloplasmin, changes of Cu plasma levels are faster than plasma ceruloplasmin levels, and so serum Cu would provide more accurate information.

To calculate statistical significance we use the NCSS software for T tests for two groups, by comparing the means and standard deviation, also Pearson correlation, and Wald Z test for proportions. Quantitative data are expressed as mean  $\pm$  standard deviation.

### Treatment administered

The active treatment administered is a combination of spirulina, curcumin and boswellia extracts, 400-50-50 mg per capsule, which were prepared alongside matching placebo capsules by DVR Pharm SRL and BioNovativ SRL.

Spirulina is the common generic name for about 15 species from two genres of monocellular microalgae (Spirulina and Arthrospira), most common being Arthrospira platensis (11), which grows naturally in freshwater and saltwater in all continents and most climates, optimally in an alkaline milieu (pH 9.5-12). It was used as the only source of nutrition in some areas in Africa for more than 1 month at a time and its first documented use was in China around 2000 years ago also during periods of famine (12). It has a very high protein content: 40-65% of dry mass depending on species and conditions of growth/cultivation (13), it has all essential amino acids, minerals (Fe, K, Na, Ca, P, I, Mg, Zn, Cu, Se, Mn, Cr) and vitamins (carotenoids 5 mg/g vitamins B1, B2, B3, B6, B12, folate, biotin, pantothenic acid, vit K1, K2), gamma linolenic acid, chlorophyll and phycocyanin, thus being a complete food source.

Alongside this remarkable composition it has important quantities of antioxidants, such as superoxide dismutase (360 UI/g), zeaxanthin (3 mg/g), vitamin E (14).

Curcumin is natural extract from the roots of Curcuma longa, has also been in use for centuries in Asia for various ailments. It is a potent anti-inflammatory substance with pleiotropic actions, including antioxidant and metabolic modulation, and its effects are well studied, with multiple clinical trials showing benefits in arthritis, inflammatory bowel disease, prediabetes, and even in early stages of neoplastic disease. A recent meta-analysis on 15 randomized clinical trials shows that curcumin down-regulates inflammation and oxidation products by reducing levels of IL-6, hs-CRP, and MDA (15).

Boswellia is also a potent anti-inflammatory molecule by blocking the NFkB pathway and modulating the activity of regulatory and T-cell (16). Together with Curcuma it was shown to inhibit production of inflammatory cytokines IL-6, IL-8, TNF- $\alpha$  and reactive oxygen species (17).

**Patients are instructed to take two capsules twice daily, about 30 minutes before breakfast and lunch.**



**Any and all adverse reactions are to be promptly reported to the study investigator.**

Benign thyroid nodules are among the most common endocrine disorders. Recent advances in diagnostic imaging and pathology have significantly contributed to better risk stratification of thyroid nodules. However, current treatment options, beyond surgical approaches are limited. The following placebo-controlled study presents, to the best of our knowledge, the first results of a non-invasive therapy for benign thyroid nodules.

The efficacy and safety of a supplement containing spirulina, curcumin and boswellia in euthyroid patients with benign thyroid nodules, is assessed by a 3-month, double-blind, placebo-controlled study. Patients with benign, single thyroid nodules with largest diameter of less than 5 cm are evaluated in a prospective placebo-controlled cross-over trial, across 12 weeks (3 visits with six-week intervals). At each visit, the target thyroid nodule was recorded in two dimensions. In addition, plasma levels of thyroid stimulating hormone (TSH), free thyroxine (fT4) and copper (Cu) are assessed.

### **Study Summary**

Design: double blind, placebo controlled, crossover

#### **Inclusion criteria:**

- adult F / M, euthyroid patients
- thyroid nodules documented ultrasound, without malignant suspicion (optimum 10-25 mm)

#### **Exclusion criteria:**

- clinical suspicion of hyperthyroidism / Hashimoto
- ATPO increased
- TSH, T3, T4 in abnormal range

Three clinical and laboratory assessments (**study visits V-V3**) are made for each patient:

- initially (upon enrollment in the study) - V1,
- after the first treatment (6 weeks after enrollment) – V2
- final (12 weeks after enrollment) - V3

Thyroid ultrasound and TSH, fT3, fT4, serum copper analysis are performed at each of these study visits.

The treatment administered to each patient consists of 4 capsules / day, administered 2 + 2, with ½-1 hour before breakfast and lunch. Some capsules - supplement A - contain spirulina (500 mg) + curcumin (50 mg) + boswellia (50 mg), - and others are placebo - supplement B;

Each patient has 2 treatments of 6 weeks, each patient has both the active preparation and placebo, and the sequence is type and sequence of capsules is blinded to both the investigator and the patients.

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The size of the nodules is evaluated (by measuring the largest diameters) after 6 weeks with the active substance, about 35 patients to make the 3 study visits.

### Study Schedule

Enrollment/Initial visit		Visit 2		Visit 3 - final
Thyroid Ultrasound	6 weeks between V1 and V2	Thyroid Ultrasound	6 weeks between V2 and V3	Thyroid Ultrasound
Laboratory TSH, fT3, fT4, seric Cu		Laboratory TSH, fT3, fT4, seric Cu		Laboratory TSH, fT3, fT4, seric Cu
A / B supplement administration		A / B supplement administration		Clinical Observations

There are three visits for each patient, noted as V1 – initial visit at enrollment; V2 – intermediary visit, six weeks post-administration of placebo (P) or active ingredients (AI); and V3 – final visit, after another 6 weeks of administration of P or AI. At each visit, each patient was tested for plasma TSH, fT4 and Cu levels, by an independent laboratory and a thyroid ultrasound was performed by an endocrinologist (DM) using the same ultrasound machine; the nodule images and dimensions were printed for comparison.

At V1, patients were randomly selected to receive either AI or P, so that half of them (n=17) received P and the other half (n=17) received AI. Subsequently at V2, the 17 patients previously on P received AI, and 12 of the 17 patients previously on AI received P, while the remaining 5 randomly chosen patients continued to receive AI. Consequently at V3, 17 patients received the sequence P-AI, 12 patients received AI-P, and 5 patients had AI-AI. The study was registered on clinicaltrials.gov site with Protocol ID “TiroNod” and ID number NCT03535974, and titled: Efficacy of a Spirulina Supplement for Amelioration of benign thYroid nodules (ESSAY).

### Statistical analysis plan

NCSS 16 software is used for data analysis. Quantitative data were expressed as mean  $\pm$  standard deviation (SD). Paired sample T test was used for comparisons of two groups (AI and P). Pearson’s correlation was used to assess correlations between specific variables. Wald Z test for proportions was used for comparing differences in outcomes between two groups. One-way ANOVA test with the Tukey and Bonferroni post-hoc can be used to compare values of TSH, fT4 and Cu.  $\alpha=0.05$ , and  $P<0.05$  indicated statistical significance.

Total patients enrolled, mean age, distribution by sex

Total completing the study and are evaluated for the primary outcome - the size of the thyroid nodules evaluated by ultrasound at 1<sup>st</sup> (initial), 2<sup>nd</sup> (intermediate) and 3<sup>rd</sup> (final) patient visits. These patients will also be tested for serum TSH, fT4 and Cu.

The overall reduction in the measured surface of the thyroid nodules for all the study participants, calculated as the mean percentage decrease in the surface of nodules (V3-V1),

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For all patients, mean initial nodule area at initial visit (V1 S) vs 2<sup>nd</sup> visit (V2 S) and at 3<sup>rd</sup> visit (V3 S) mean nodule area

From the total patients with the 3 ultrasound evaluations, the percentage (%) who had decreases in the areas of thyroid nodules of 5% or more at the end of the study, and percentage (%) with decreases of 10% or more of the thyroid nodule area at V3.

Percentage change from initial vs. final area of thyroid nodules, number of patients in groups

Initial vs. final area decrease %	< 0 (increase)	~ 0 (unchanged)	5-9.9% (decrease)	10-19.9% (decrease)	20-49.9% (decrease)	50-99% (decrease)
Number of patients						

A comparison of the effects of the active substance vs. placebo on decreasing the surface of thyroid nodules that after the initial administration of the placebo and active substances (V2-V1 for all patients), the mean decrease with AI vs the mean decrease with placebo.

Comparing effects for the two groups (AI and P) is done with two-sided T-test for the two groups.

Additionally, we will use two statistical tests: the Wald Z test to compare the proportion of patients with modifications in nodule areas, grouped in AI vs. P and also tested both the equal variance and Aspin-Welch variants for the difference of means and SD between the AI and P groups.

Will also calculate the difference in the proportion of patients with decreased thyroid nodule areas on active substances vs.. placebo groups.

For the overall effects of the placebo and the active substances on all administrations, we compare the effects of the administration of the active substances by the mean decrease in nodule areas, versus the mean decrease of nodules following placebo administration.

Testing for statistical significance between the two groups (active and placebo) at V3 versus V1 with T-test for means and standard deviation, calculated with the equal variance T-test and the unequal variance Aspin-Welch test.

Comparing the means, minimum and maximum values nodule areas in the AI (Act) and P (Plac) groups after the initial 6 week administration of AI or P (V2-V1, noted "A") and after 12 week of AI or P (V3-V1, noted "A+B", A being the initial 6 weeks, B the final 6 weeks of study).

Beyond efficacy, we are monitoring the influence of treatment on thyroid function and the correlation with Cu levels.

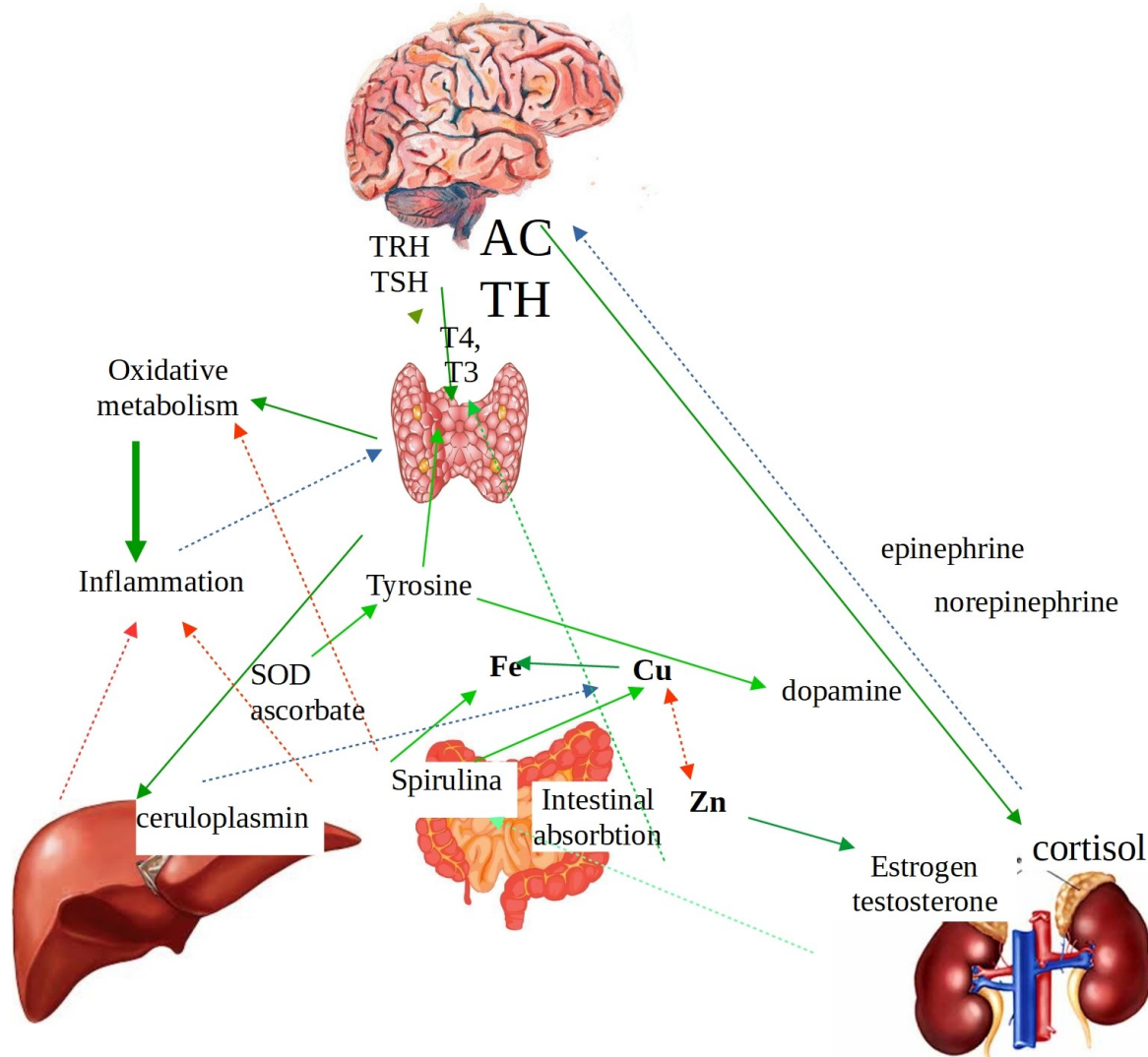
To determine whether the active substances or the placebo had a significant effect on thyroid function, we compared the respective values of TSH and fT4 for all 3 visits (visit 1 vs.. 2, visit 1 vs.. 3 and 2 vs.. 3) with the one-way ANOVA test and the Tukey and Bonferroni post-hoc tests.

To verify the interdependence of the four variables measured in this study: nodule surface, TSH, fT4 and Cu levels, we calculate the Pearson correlation indices between these variables.

The link between thyroid function and ceruloplasmin, the most important copper-based enzyme, was observed a few decades ago, with a significant elevation of serum ceruloplasmin being observed in patients on oral contraceptives together with an increase in thyroxine binding index, while free thyroxine levels were unchanged (20). Similarly, in male rats estradiol elevated ceruloplasmin levels, while testosterone and thyroid hormones inhibited this action (21). Furthermore, it was shown that administration of L-thyroxine suppresses the stimulation of ceruloplasmin by estrogen administration (22). Opposite modifications of ceruloplasmin levels in hypo- and hyper-thyroid patients, in tandem with thyroid function, as well as oxidation status (malonyldialdehyde) and uric acid levels were observed by a Romanian team (23). New direct links between ceruloplasmin, oxidative stress and inflammation were recently shown with TNF-related apoptosis-inducing ligand (TRAIL) down-regulating ceruloplasmin expression in inflammation (24), and modulation of TNF- $\alpha$ -induced neutrophil apoptosis by ceruloplasmin (25). Low levels of ceruloplasmin were shown in a hypothyroid patient with Hashimoto thyroiditis (26).

Recent findings have shown that thyroid hormones increase serum copper levels by stimulating the synthesis of ceruloplasmin in the liver as reflected in the mRNA levels, and this action is abolished in cases of hyperthyroidism associated with receptor resistance to thyroid hormones (27). In addition, the ratio of serum copper (Cu) and selenium (Se) was proposed as an indirect marker for receptor resistance to thyroid hormones. Stimulation of hepatic ceruloplasmin mRNA by thyroxine and dexamethasone was previously shown in rats (28). It was also found that a copper-deficient diet increases the levels of serum free triiodothyronine (fT3) but not free thyroxine (fT4) in rats (29); similarly a strong link between serum T3 and Cu was demonstrated in rat pups (30). Cu and Zn levels in serum and tissue are modulated by thyroid hormones as shown by thyro-parathyroidectomy and T4 replacement in rat. Interestingly, it was seen that ceruloplasmin is present in follicular carcinoma and absent in adenomas (31-33). Further differences in the oxidative and inflammatory processes involved in benign and malignant thyroid nodules versus normal thyroid tissue (hypoxia-inducible factor-1, complement factor D, matrix metalloprotease 1, Von Willebrand Factor - were shown in different studies (34, 35) The interrelations between thyroid hormones, ceruloplasmin, adrenal hormones and important metabolic processes are presented in Fig. 3.

Figure 3. Interactions between thyroid hormones, ceruloplasmin, adrenal hormones, and processes of intestinal absorption, oxidative metabolism and inflammation: T3, T4 – triiodothyronine, thyroxine, TRH/TSH – thyroid releasing/stimulating hormones, ACTH – adrenocorticotrophic hormone, SOD – superoxide dismutase,



In summary, the combination of spirulina-curcumin-boswellia is effective in reducing the size of benign thyroid nodules and can be safely administered in the doses used in the presented clinical study. The mechanism by which the decrease of the nodule size is facilitated, is probably linked to the anti-inflammatory effects, the improvement of the antioxidant status and the ceruloplasmin actions. However, additional studies are needed in order to elucidate these associations, which may also provide important insights into the pathogenesis of thyroid nodules. or applicable.

**Funding**

This study is financed by a grant received by The Bio-Forum Foundation from DVR Pharm SRL and BioNovativ SRL which intend to manufacture and bring to market the spirulina-curcumin-boswellia combination under the name "TiroDren".

**Ethics approval and consent to participate**

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All participants will provide written informed consent for the whole study. The study observes the ethical rules on clinical studies established by the Institute according to the World Health Organisation guidelines.

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