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RESEARCH ARTICLE

Antitumor Activity of Selenium-Enriched *A. Platensis* Combined With Polyphenols, Vitamin E and SOD

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Abstract

Background: *Arthrospira Platensis*, widely used as a nutritional supplement, can accumulate selenium (Se) in a dose and time-dependent manner, a trace element with potential anticancer effects. In this study, we were interested in the cytotoxic effect of Se either as inorganic or organic form, in Se-enriched *A. platensis*, and in association with an antioxidant cocktail (polyphenols (olive oil extracts), vitamin E and superoxide dismutase) (ULTRASELEM® formulation).

Methods: Different *A. platensis* enrichment protocols of Se were assessed and the antitumor activity of the different forms of Se or in the ULTRASELEM® formulation was tested *in vitro* on a murine epithelial colorectal adenocarcinoma (CT26) cell line.

Results: Single dose Se enrichment protocol provided a more profitable Se-enriched *A. platensis* than daily Se enrichment. Then Se-enriched *A. platensis* in ULTRASELEM® induced significant *in vitro* activity on CT26 cells with 30% of cell growth inhibition with two ULTRASELEM® capsules (50µg.L-1 Se) and more than 90% inhibition with eight capsules (200µg.L-1 Se) without reaching maximal daily recommended Se intake.

Conclusion: This ULTRASELEM® formulation represents an original combination of antitumor and antioxidant properties eliciting promising efficacy on cancer and thus an excellent candidate for therapeutic use in cancer prevention and treatment.

Keywords: Selenium, *Arthrospira Platensis*, *Spirulina,* ULTRASELEM®, anticancer agent, colorectal cancer

Introduction

Colorectal cancer represents one of the leading cause of worldwide mortality. Nowadays, the use of natural compounds with antitumor activity represents a challenging way to fight against cancer. Selenium (Se) is an essential micro-nutriment with several functions in anti-inflammatory processes [1] thyroid hormone metabolism [2] and antioxidant mechanisms [3-5] several *in vitro* and pre-clinical studies demonstrated anticancer and chemopreventive actions of Se in particular with colorectal cancer [6-9]. Moreover, low Se plasmatic levels were associated with increased risk of colorectal cancer development [10]. Besides, inorganic Se is weakly assimilated by organisms, in comparison to its organic form, hence, it is of great interest to developp organic Se for dietary supplementation. In fact, it has been demonstrated an enhanced efficiency of Se-enriched aliments, probably due to a better Se bioavailability and/or different metabolic conversion [11].

Arthrospira Platensis, also known as *Spirulina*, was reported as a good candidate for selenium (Se) enrichment and a promising source for dietary Se supplementation [12, 13]. This cyanobacterium (blue-green microalga) was used for decades as alimentation source due to its high nutritional properties with enriched proteins, carbohydrates, minerals, vitamins, fibers and pigments [14, 15]. Moreover, several studies have reported interesting antibacterial [16], antiviral [17-19] and antioxidant effects [20] of *Spirulina*. In addition, we have shown earlier, that Se-enriched *A. platensis* had interesting antioxidant effect and a potentiating antitumor activity when combined to antitumor drugs [21]. Aside, several studies suggested that Se could have synergic antitumor potency in association with vitamin E [22, 23] and polyphenols [24]. Therefore, the aim of the study was to evaluate the antitumor activity of Se-enriched *A. platensis* combined with polyphenols (olive oil extracts), vitamin E and superoxide dismutase (SOD) (ULTRASELEM®, patent FR2947179A1) on colorectal cancer *in vitro*.

Material and Methods

Platensis Enrichment

A. platensis was grown as previously described by Zarrouk [25]. Biomass density was determined by absorbance reading at 750 nm [26]. Enrichment with sodium selenite (Na_2SeO_3,

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Sigma-Aldrich) started when *A. platensis* density reached 2 g.L-1 and was carried out for seven days according to our previous report [21]. Two different protocols were used for Se enrichment, a single Se addition the first day (0.15 g.L-1) and a daily Se addition during seven days (0.15 g.L-1x7 days). Enriched microalgae were then used for cell treatment either directly or after filtration through a 0.2µm filter, or as ULTRASELEM® formulation (Vita-Api, La Seyne sur Mer, France). Measurement of Se enrichment into *A. platensis* was realized by inductively coupled plasma mass spectrometry (ICP-MS) by Eurofins Analytics (Nantes, France).

UTRASELEM® composition

ULTRASELEM® was composed of dried Se-enriched *A. platensis* obtained according to the procedure described above and Se enrichment reached 70 mg Se.Kg-1, according to the process described in the French patent applications FR1257009 and FR2947179A1. Then the powder was supplemented with polyphenol (70 g.Kg-1 olive extract, 70 g.Kg-1 Grape polyphenol and resveratrol), vitamin E (14 g.Kg-1) and melon SOD (14 g.Kg-1) cocktail.

Cancer cell viability assay

The murine colon cancer cell line (CT26.WT) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 medium (R8758, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Dutcher), 100 U.mL-1 Penicillin/streptomycin (Sigma-Aldrich), 2.5 µg.mL-1 amphotericin B (Sigma-Aldrich) and 0.5 mM L-Glutamin (Sigma-Aldrich) at 37°C and 5% CO_2 in a humidified atmosphere. Cancer cell viability was evaluated using the 3-(4, 5-dimethythiazol-2-yl)-2, 5-phenyltetrazolium bromide (MTT) assay as previously described [21]. Briefly, cells were seeded in 96-well plates at the density of 103 cell/well and after 24h; they were incubated with different concentration of sodium selenite or *A. platensis*, Se-enriched *A. platensis* or ULTRASELEM® biomass for 72h. Each condition was performed at least in triplicate and repeated three times.

Statistical analysis

Data analysis was performed using SPSS 17.0 software package (SPSS Inc, Chicago, IL, USA). All data were expressed as means ± standard error of the mean (SEM). Comparison between different groups was analysed with One-way ANOVA test. Concentration-dependent inhibition of cell growth (IC50) was determinated by nonlinear regression.

Results

Effect of different preparations of Se-enriched *A. platensis* on cancer cell viability

Colorectal cancer CT26 cells were treated *in vitro* with different preparations of *A. platensis* or *A. platensis* enriched either with a single dose of Se (protocol 1, addition of 0.15 g.L-1) or a daily Se enrichment (protocol 2, addition of 0.15 g.L-1 x7). Then, *A. platensis* biomass was either filtrated as detailed in material and methods or not. Firstly, non–enriched *A. platensis* filtrated or not, did not affect CT26 cell survival (Figure 1A and 1B). When CT26 cells were incubated with filtrated *A. platensis* preparations, we observed mild but

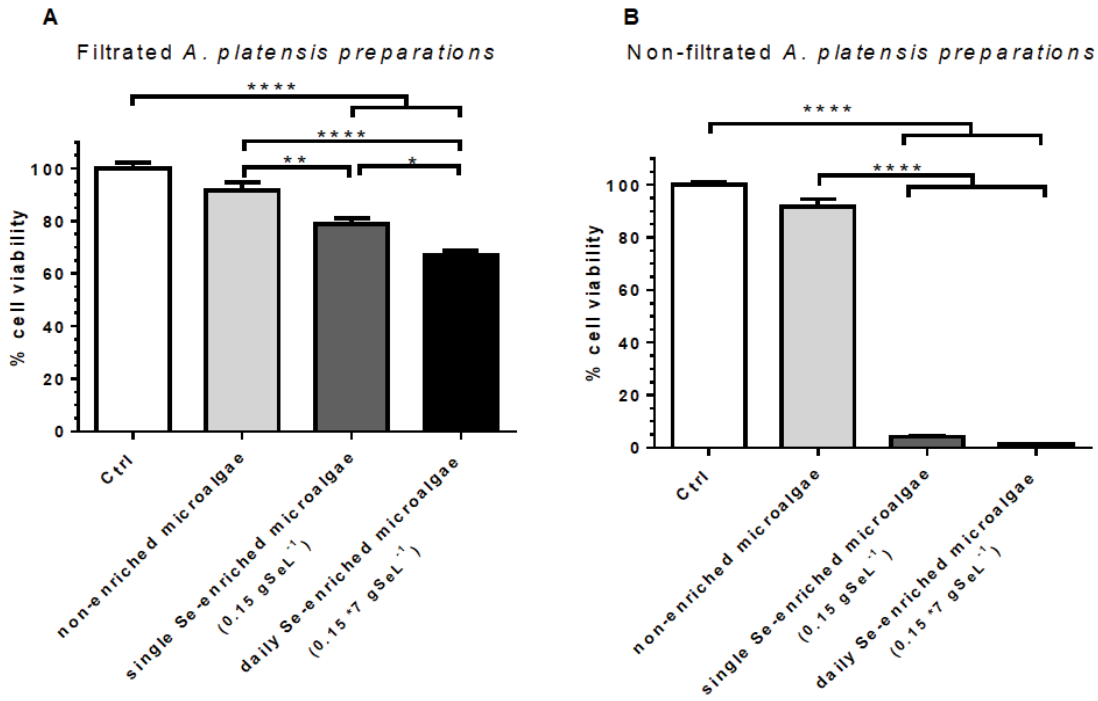

Figure 1: Effect of different preparations of Se-enriched *A. platensis* on cancer cell survival. CT26 cells were treated with 0.2 g.L-1 of filtrated (A) or non-filtrated (B) *A. platensis* enriched, or not, with Se. Se enrichment of microalgae was realized with a single (0.15g.L-1) or a daily (0.15x7 g.L-1) Se addition; Data are presented as mean ± SEM; *p<0.05, **p<0.01, ****p<0.0001

significant reduction of cell survival for groups treated with *A. platensis* enriched according to protocol 1 (78.8%) and 2 (66.8%), in comparison with cells treated with non-enriched microalgae or control untreated cells. Surprisingly, when non-filtrated preparations of enriched *A. platensis* were used, cell survival was significantly reduced by more that 95% for the two enrichment protocols (cell survival = 4.2 and 1.4 % for protocol 1 and 2 respectively) (Figure 1B).

Measurement of organic Se absorbed by Se-enriched *A. platensis* and non-absorbed inorganic Se concentration in filtrates

To explain the important variations of antitumor efficacy obtained between filtrated and non-filtrated preparations of Se-enriched *A. platensis*, intracellular Se concentrations (organic Se) contained into *A. platensis* and extracellular Se concentrations (inorganic Se) in filtrates were measured after five and seven days of enrichment according to the two protocols. As summarized in table 1, organic Se contained into *A. platensis* was very different following the 2 protocols; Se intracellular concentration was of 70 ± 7 mg.Kg-1 and 188±56 mgKg-1 after protocol 1 and 2 respectively. While de dose of enrichment was 7 times higher with the protocol 2 compared to the protocol 1, the enrichment was only of 2.6 times superior. These results suggest that an important sodium selenite (inorganic Se) concentration was not absorbed by *A. platensis* and transformed in organic Se and remains in suspension, which could explain the important cell inhibition obtained with non-filtrated *A. platensis* treatment in comparison with filtrated one (Figure 1). In fact, Se concentration was estimated in Se-enriched *A. platensis* filtrate after the two enrichment protocols to evaluate the non-absorbed concentration of Se which still in suspension at inorganic form. Se concentration in filtrate at the end of enrichment (7days) was equivalent to 70±9 mgL-1 and 440±88 mgL-1 after protocol 1 and 2 respectively.

Cytotoxic efficacy of inorganic Se

Aside, CT26 cells were treated with different concentrations of inorganic Se (sodium selenite). The half-maximal inhibitory concentration (IC50) was obtained at a Se concentration equivalent to 480 µg.L-1 (Figure 2). According to these results, we can notice that inorganic Se concentrations measured in Se-enriched *A. platensis* filtrate for the two-protocol enrichments (Table 1), induced more than 95 % mortality.

Intracellular organic Se (mg.Kg-1)			Inorganic in filtrate (mg.L-1)	
	Day 5	Day 7	Day 5	Day 7
Protocol 1	31.6 ± 9.5	70 ± 7	75.0 ± 15	70 ± 9
Protocol 2	60.8 ± 18.2	188 ± 56	500 ± 100	440 ± 88

Table 1: Measurement of Se in Se-enriched *A. platensis* after enrichment protocols.

Intracellular organic Se concentration and inorganic Se in filtrates were determined by inductively coupled plasma mass spectrometry as described in material and methods after 5 and 7 days of the 2 protocols of enrichment

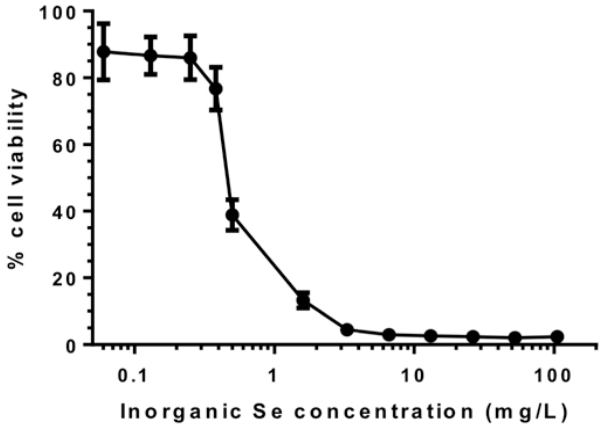

Figure 2: Effect of sodium selenite (inorganic Se) on CT26 cell viability. Viability was measured using MTT assay after incubation with Se for 96h. The experiments were performed in triplicate, and the data are the mean ± SD

These results confirm our results obtained with cell treatment with non-filtrated enriched *A. platensis* shown in (Figure 1B).

Effect of ULTRASELEM® on cancer cell viability

Se-enriched *A. platensis*, used for ULTRASELEM®, was enriched according to the first protocol (single dose of 0.15 g.L-1 Se). Furthermore, in Se-enriched *A. platensis* was added a vitamin E, polyphenols and SOD cocktail to obtain ULTRASELEM®. In order to assessed the cytotoxic effect of the mixture, CT26 cells were treated with different concentrations of ULTRASELEM® according to the procedure described in materiel and methods. Then, we observed a dose-dependent reduction of cell viability of CT26 cells (Figure 3). The half maximal inhibitory concentration (IC50) was obtained at 1.48 g.L-1 of biomass (corresponding to 103 .6 µ g . L -1 Se). Furthermore, interestingly, the recommended daily dietary

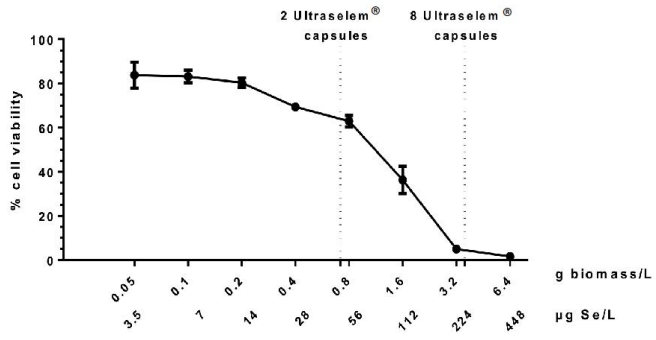

Figure 3: Effect of ULTRASELEM® on CT26 cell viability. Cells were treated with different concentrations of ULTRASELEM® biomass. Corresponding Se concentration was reported for each biomass concentration. The equivalent biomass of 2 and 8 capsules of ULTRASELEM® capsules was reported on the graph. Viability was measured using MTT assay after incubation with ULTRASELEM® biomass for 96h. The experiments were performed in triplicate, and the data are the mean ± SD.

intake ofdietary intake of 50µg.L- 1 Secorresponding to two capsules of ULTRASELEM ® reduced cell viability by 30% while increased dosage of Se >200 µg.L-1 , corresponding to eight capsules of ULTRASELEM ® was able to induced more than 90% of cell growth i nhibition.

Discussion

In this study, we were interested in the cytotoxic effect of Se either as inorganic or organic form, in Se-enriched *A. platensis*, and in association with an antioxidant cocktail (ULTRASELEM® formulation). Our results reported significant *in vitro* colon cancer cytotoxicity of Se-enriched *A. platensis*. We tested two different enrichment protocols and we observed that single Se enrichment protocol (addition of 0.15 g.L-1) provided a more profitable Se-enriched *A. platensis* than daily Se enrichment. We also reported very high cytotoxicity of inorganic Se. Nevertheless, because of its low bioavailability and potential toxicity in comparison with organic Se, Se-enriched *A. platensis* was used for ULTRASELEM® formulation. This formulation also included a cocktail of vitamin E, polyphenols and SOD, with interesting antioxidative effects. We could observe that ULTRASELEM® induced cytotoxic *in vitro* activity on colon cancer cells with 30% of cancer cell growth inhibition with two ULTRASELEM® capsules equivalent to 50µg.L-1 of Se and more than 90% inhibition with eight capsules (200µg.L-1 Se) without reaching maximal daily recommended Se intake.

Recommended dietary allowance of Se is 55 µg/day (for 14 years or older), with a tolerable upper intake limit of 400 µg/day [27]. Low Se intake status has been associated with increased risk of mortality, poor immune function and cognitive decline [2]. Moreover, several clinical trial interested on whether Se influences development of adenocarcinoma by supplementation with Se alone [7, 28], or in association with other antioxidant nutrients [29, 30]. Some studies revealed a significant protective effect on colorectal adenoma recurrence while some other studies failed to observe any association. These discrepancies could be explained by the differences in subject baseline Se status, as reported by studies which associate a lower Se levels, caused by low Se availability, with a higher cancer risk [10]. Selenium and vitamin E Cancer Prevention Trial (SELECT) did not report any preventive effect of Se and vitamin E supplementation on prostate and other cancers [22, 30]. These results were attributed in part to a higher basal Se of participants [11]. Besides, the Nutritional Prevention of Cancer intervention trial (NPC) reported significant effect of Se supplementation on colorectal carcinoma risk only in subjects with a baseline plasma Se < 106 µg.L-1 [32]. Such association was also observed with European prospective investigation of cancer and nutrition cohort [33]. In fact, variations in baseline Se status are principally due to differing soil Se levels and resulting in food content that engender great variation in dietary Se intake worldwide. Se-poor soils are characteristics in several European countries and some regions in China, whereas high Se content is common in North and South American [10, 11]. These results highlight the interest of Se supplementation for low Se basal subjects as European populations.

We showed that Se-enriched *A. platensis* associated to an antioxidant cocktail had a significant cytotoxicity on colorectal cell lines, in comparison with non-enriched microalga, as we previously reported on other different cell lines [21]. Such results were also noticed *in vivo* with Se-enriched yeast [6]. We also reported important cytotoxic effect on cancer cell viability of sodium selenite alone which is in accordance with previous works [34, 35]. Nevertheless, one of the problems of the use of inorganic Se is the threshold between toxicity and therapeutic efficacy. In fact, studies have reported that inorganic form induces single strand breaks DNA, and cell death by apoptosis [36] whereas organo-selenium compounds, even at high level of Se, can cause cell death by apoptosis without evidence of DNA single strand breaks [37, 38]. In fact, once enriched with Se, nutriments could transform it into various organic Se compounds as the Seleno-methionine (SeMet), the main organic Se form accumulated by microalgae, which is considered as a potent anti-carcinogen [39]. In contrast to inorganic Se, organo-selenium compounds can be tailored to achieve greater chemopreventive efficacy with minimal toxic side effects by structural modifications. Moreover, they present a higher bioavailability and different cellular effects from those elicited by inorganic form [11, 40]. It was also suggested that Se-enriched nutriments had more beneficial effects than organic Se compounds [41]. This could be explained by a better Se bioavailability and/or different metabolic conversion [11]. In fact, in Se-enriched foods, the Seleno-methionine is protein-bound, which confer a better protection from oxidation than when exposed to air in the pure state [42]. This protection could also play an important role in Se uptake, excretion, tissue distribution, transport and metabolism and was proposed as a reason explaining the failure of SELECT trial, which used organic Se supplementation rather than Se-enriched aliments as the NPC clinical trial [11, 42]. Moreover, Se-enriched nutriments contain several organo-selenium compounds, other than Seleno-methionine, which would confer stronger anti-oxidative and potentially chemopreventive effects [43].

Several possible mechanisms have been proposed to explain the protective role of Se against colorectal cancer development as induction of apoptosis [38], increased immune function [44] and protection from oxidative DNA damage [45]. In fact, Reactive oxygen species (ROS) are reported to be one of the principal sources of endogenous DNA damage that has been widely accepted as a major cause of cancer [46]. Therefore, antioxidant agents that lower ROS could be chemopreventive by reducing genotoxicity and by slowing cancer progression [47]. Interestingly, we observed in this study a potentiated antiproliferative activity of ULTRASELEM® where Se-enriched *A. platensis* was associated to an antioxidant cocktail (vitamin E, resveratrol and SOD). Se-enriched *A. platensis* is able to incorporate part of the Se into proteins specifically via Se-Cys insertion machinery and thus synthetizes various selenoenzymes as glutathione peroxidase, a well-known antioxidant enzyme [39]. In addition, antioxidant activities of

Se-enriched *A. platensis* have already been reported [21, 48]. Vitamin E functions as the major lipid-soluble antioxidant in cell membranes. It is a chain-breaking, free radical scavenger and inhibitor of lipid peroxidation, specifically biologic activity relevant to carcinogen-induced DNA damage [31].

Furthermore, Resveratrol, a polyphenol found in grapes and wine, is a natural compound with chemopreventive effects in different systems based on its striking inhibition of diverse cellular events associated with tumor initiation, promotion and progression [49]. This is partly attributable to its strong anti-oxidant activity and its protection against lipid peroxidation within cell membranes and damage to DNA resulting from ROS [50]. Aside, SOD plays an important role in protecting the organism against the damaging effects of the superoxide radical through converting it to hydrogen peroxide [51]. Several *in vitro* and *in vivo* studies in addition to clinical trials tested efficiency of Se or Se-enriched nutriment with one or more of these compounds. *In vitro* studies reported synergic effects of Se and vitamin E in inhibiting tumor growth [22, 52, 53], and animal models studies had also shown beneficial effect in suppressing carcinogenesis and without adverse interactions in addition to a reduced level of oxidative DNA damage more than did either agent alone [54]. The SELECT study, associating Se and vitamin E supplementation, did not report any positive effects on cancer prevention but failure were attributed to the nature of supplementation and Se baseline population [11]. A Chinese clinical trial, the Lixian study, reported significantly decreased cancer mortality and a lower incidence of oesophageal/gastric cancers with a supplementation combining Se-enriched yeast, vitamin E and β-carotene [56, 57]. The SU.VI.MAX study, associating Se-enriched yeast, vitamin E, vitamin C, β-carotene and zinc, reported a moderate non-significant reduction in prostate cancer rate [58]. Moreover, antitumorigenic activity of a mixture of Se, polyphenols of green tea in addition to ascorbic acid, lysine, proline, arginine, N-acetyl-cysteine, copper and manganese were tested and showed anti-proliferative and anti-invasive effects on human colon cancer cells [59] and breast cancer cells [60]. A synergic effect was observed between the different mixture components enhancing thus the antitumorigenic effects [61].

In this study, we have demonstrated an interesting antiproliferative effect of Se-enriched *A. platensis* associated with vitamin E, polyphenols and SOD on colorectal cancer cell line *in vitro*, which could be attributed to the antioxidant synergic efficiency of these compounds. Interestingly, we observed a dose dependent efficacy of ULTRASELEM®. In fact, two ULTRASELEM® capsules induced a moderate antitumor efficiency. This dose corresponds to the daily-recommended Se intake. The combined antioxidant effects of Se, vitamin E, polyphenols and SOD, could explain the antitumor efficacy. Moreover, maximal daily-recommended ULTRASELEM® corresponding to eight capsules, provided an important Se intake without reaching the daily maximal dose, displayed a high cytotoxic effect, which reached 90% of cancer cell growth inhibition. Therefore, a daily ULTRASELEM® intake of eight ULTRASELEM® capsules could thus play an important role for cancer prevention and treatment. To conclude, this formulation represents an original combination with promising antioxidant and antitumoral effects on cancer and thus an excellent candidate for therapeutic use in cancer prevention and treatment. *In vivo* and clinical studies are now required to confirm our results.

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Author Contributions

S.D research data/wrote/edited manuscript, H.O designed experiments and reviewed the manuscript, C.R. designed experiments, research data/wrote/edited the manuscript and is the guarantor of this work and as such, had full acces to all the study and takes responsibility for the data integrity and the data analysis accuracy.

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