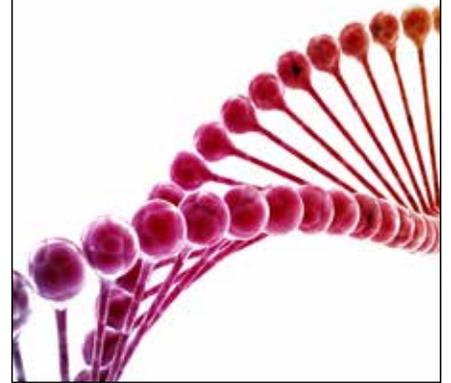




Products Clinical Study:

Epigenetic Study

Paul Davis, Ph.D.¹,
Catherine Davis, Ph.D.¹,
& Dan Gubler, Ph.D.²



SUMMARY:

A gene can be thought of as a switch that turns on and off different processes in the body. The turning ON and OFF of genes that support different health outcomes is known as epigenetics. Good lifestyle habits, like a nutritious diet and proper exercise, can help maintain proper epigenetics. Poor diet, sedentary lifestyle, restless sleep, pollution, and stress can cause genetic switches in the body to become mis-regulated, which can lead to poor health.

Phytonutrients, such as the ones included in THREE products, have also been shown in scientific literature to maintain healthy epigenetic function in the body. The purpose of this research study was to determine whether science based nutritional supplements containing a range of phytonutrients in highly bioavailable delivery forms, could maintain key genes important in supporting human health.

GENES TESTED:

- *nrf1*—assists in maintaining healthy energy levels and cellular health.
- *nfKb*—supports healthy inflammatory status, deactivation of this gene is desirable in supporting your health.
- *ppara*—responsible for producing ATP, the energy molecule of the cell. Supports human health.
- *sod1*—supports healthy antioxidant status, important for maintaining health.
- *sirt1*—helps maintain energy production, supports cellular function, and promotes brain health.
- *glp1*—helps support healthy blood sugar and blood insulin levels.
- *bdnf*—helps maintain healthy cognitive function, activation of bdnf supports human health.
- *il6*—helps maintain a healthy inflammatory status, low *il6* levels desirable for promoting human health.

STUDY RESULTS

Four nutritional supplements developed by THREE were tested. The results were shown to have a positive impact on regulating or suppressing certain gene functions in supporting the desired health outcomes. See the charts and read through the full study below!

¹ Paul Davis, Ph.D., Catherine Davis, Ph.D, Trinity Bioactives Ltd

² Dan Gubler, Ph.D., THREE International LLC



Maintenance of Healthy Epigenetic Function in Human Cells by THREE Products

Paul Davis, Ph.D.¹, Catherine Davis, Ph.D.¹, and Dan Gubler, Ph.D.²

Introduction:

The human body consists of 20,000 genes that maintain the millions of chemical reactions performed therein. A gene is a segment of DNA that encodes a protein. The turning ON and OFF of genes that support different health outcomes is known as epigenetics. Good lifestyle habits, like a nutritious diet and proper exercise, can help maintain proper epigenetics. Conversely, poor diet, sedentary lifestyle, restless sleep, pollution, and stress can cause genetic switches in the body to become mis-regulated, which can lead to poor health.

Phytonutrients are molecules in plants that can help maintain human health. In addition to supporting a healthy antioxidant status, phytonutrients have also been shown in scientific literature to maintain healthy epigenetic function in the body. The purpose of this research study was to see if science based nutritional supplements, containing a wide range of phytonutrients in highly bioavailable delivery forms, can maintain key genes that are important in supporting human health. If this is the case, it would represent a new and novel mechanism of action by which nutritional supplements help to maintain health and wellness.

The aim of this project was to investigate the effects of four nutritional supplements developed by THREE International (Vitalité, Revive, Éternel, and Collagène) on their ability to help regulate important genes in the body. The genes tested include the following:

- *nrf1* — Assists in maintaining healthy energy levels in the body along with supporting cellular health. It is good for *nrf1* to be activated in promoting proactive wellness.
- *nfkb* — Responsible for supporting a healthy inflammatory status in the body. Deactivation of this gene is desirable in maintaining health and wellness.
- *pparα* — This gene is involved in the production of ATP, which is the energy molecule of the cell. Activation *pparα* is beneficial in supporting human health.
- *sod1* — A gene that helps to support a healthy antioxidant status in the body. Activation of *sod1* is important for maintaining health.
- *sirt1* — Gene involved in maintaining energy production, supporting cellular function, and promoting brain health.
- *glp1* — This gene helps to support healthy blood sugar and blood insulin levels. It is desirable that *glp1* be activated.
- *bdnf* — An important gene that is expressed in brain tissue and helps to maintain healthy cognitive function. Activation of *bdnf* is desired for supporting human health.
- *il6* — A gene that helps to maintain a healthy inflammatory status in the body. Low *il6* levels are desirable in promoting human health.



Test Samples:

Products to be tested were nutritional supplements provided by THREE International, and are as follows:

- THREE Vitalité
- THREE Revive
- THREE Éternel
- THREE Collagène

Methods:

Sample Preparation:

1. Each of the samples was prepared in the following way:
2. Samples were dissolved in DMSO at a concentration of 100 mg/ml. Only the Vitalité capsule was dissolved in solution and used in the experiment.
3. Working solutions of each test sample was prepared in the appropriate medium.

Cell Preparation:

1. The frozen THLE-2 stock cell line was removed from liquid nitrogen and immediately thawed in a 37°C water bath. The contents were then transferred to 250 ml (75 cm²) culture flasks containing 20 ml of Bronchial Complete Cell Growth Medium (BEGM) plus 10% FBS and penicillin/streptomycin. Instructions provided by the supplier were followed.

Harvesting the Cells:

1. To detach the adherent THLE-2 cells, the medium was removed from the culture flask and the adherent cells washed with PBS to remove any traces of FBS. Then 3-4 ml of 0.05% trypsin/EDTA solution was added and incubated at 37°C for 5 min until all the cells were detached. The trypsin was then neutralized by adding an equal volume of pre-warmed 0.1% trypsin inhibitor and centrifuged at 125g (500 rpm) for 7 min at 4°C.
2. The supernatant from the flask was discarded and the cell pellet from the flask re-suspended with 10 ml of pre-warmed Bronchial Complete Cell Growth Medium (BEGM). The cell number was counted and adjusted to 1 x 10⁵ cells/ml for the culturing. A total volume of approximately 32 ml of cells at 1 x 10⁵ cells/ml was required.

Cell Culturing:

1. Flat-bottomed 24-well plates, treated for cell adhesion, were used. The plates contained cells in the appropriate medium containing 10% FBS. These plates were used for the mRNA determinations. 96 well plates were used for the cell proliferation assays.
2. For the assays 720 µl of the cell suspension (1 x 10⁵ cells/ml) were plated into wells A-D1-5 giving approximately 8.0 x 10⁴ cells/well. 720 µl of the appropriate medium only was added to wells A-D6.
3. The plates were placed in the incubator for 24 hours to allow the cells to adhere to the wells.
4. After 24 hours the plates were removed from the incubator. 80 µl of the various samples were added to the appropriate wells.



5. To the wells labelled "Control" and "Medium", 80 µl of 0.5% DMSO in culture media was added.
6. The plates were incubated at 37°C in 95% Air/5% CO₂ for a total of 24 hours.
7. At the time of termination, the culture medium was aspirated from the wells. The supernatants from each replicate well were transferred to a clean 96 well plate (non-treated) and stored at -20°C.

Cell Proliferation Assay:

1. Using one plate for each cell type, after the removal of the supernatants, 800 µl of fresh medium were added to each well. 80 µl of MTT working solution (5 mg/ml) was then added to each well on the plate and incubated for an additional 4 hours at 37°C.
2. 400 µl of MTT lysis buffer was then added to each of these wells and the plate incubated for an hour at 37°C.
3. The absorbance of each well was read using the NeoSyn2 microplate reader at 570 nm.

RNA Isolation:

1. Using the other plate for each cell type, after the removal of the supernatants, the cells were washed twice with HBSS (400 µl each time), and the washings discarded.
2. The cells were then processed to isolate the total RNA following the instructions provided by the manufacturer of the kit.

Purity and Quality of RNA:

1. The absorbance at the 260 nm run was read and was a measure of the RNA concentration in µg/µl.
2. The quality of the RNA was determined from the 260 nm/280 run and 260 nm/230 run ratios.

qRT-PCR:

1. qRT-PCR was undertaken following the protocol supplied by the Sponsor.

Experimental Design:

For the cell viability and the mRNA levels, each sample was assayed in triplicate with mean values and standard errors expressed graphically. A medium only blank was also run in triplicate.

Results:

The ability of THREE's products to maintain the expression of key genes *in-vitro* is shown in **Tables 1-7**. Note that not every product was found to modulate each of the genes in question.

Product	Concentration (µg/mL)	% Change
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Revíve	50	229
Vitalité	150	224

Table 1: Expression (in % change) of the *nrf1* gene by THREE Products.

Product	Concentration (µg/mL)	% Change
Éternel	150	-345
Revíve	150	-200
Vitalité	150	-172
Collagène	150	-125

Table 2: Expression (in % change) of the *nfκb* gene by THREE Products.

Product	Concentration (µg/mL)	% Change
Revíve	50	384
Éternel	50	380
Vitalité	150	216
Collagène	150	117

Table 3: Expression (in % change) of the *pparα* gene by THREE Products.

Product	Concentration (µg/mL)	% Change
Revíve	50	185
Vitalité	50	138
Éternel	50	123
Collagène	50	122

Table 4: Expression (in % change) of the *sod1* gene by THREE Products.

Product	Concentration (µg/mL)	% Change
Vitalité	150	348
Revíve	50	293

Table 5: Expression (in % change) of the *glp1* gene by THREE Products.

Product	Concentration (µg/mL)	% Change
Vitalité	150	840
Collagène	150	570
Revíve	50	430

Table 6: Expression (in % change) of the *bdnf* gene by THREE Products.

Product	Concentration (µg/mL)	% Change
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Éternel	50	-1250
Revive	50	-123
Vitalité	50	-112

Table 7: Expression (in % change) of the *il6* gene by THREE Products.

Discussion:

Table 1 shows the results on the ability of THREE’s products to activate the *nrf1* gene. Revive and Vitalité activated *nrf1* by 229 and 224 percent respectively. According to these findings, Revive and Vitalité could help to promote natural energy production in the body.

Gene expression results for *nfkb* is shown in **Table 2**. For the body to be healthy, *nfkb* should be downregulated. Éternel and Revive strongly downregulated *nfkb* expression while Vitalité and Collagène reduced *nfkb* to a lesser extent. Based on these results, all four products tested could be expected to help maintain a healthy inflammatory status in the body.

Revive, Éternel, and Vitalité strongly upregulated the expression of the *pparα* gene while Collagène was a mild activator (**Table 3**). The body produces more energy when *pparα* levels are increased. As such, all four products tested could help to support energy production in the body.

sod1 gene expression data (**Table 4**) revealed that all four products tested were activators of this gene and should help to maintain a healthy antioxidant status in the body—which is one of the primary need states nutritional supplements focused on proactive wellness should address.

The *glp1* gene has garnered significant interest in both the scientific and nonscientific communities alike due to its involvement in supporting healthy blood insulin levels and in maintaining a healthy weight. Consequently, we were delighted when we saw that Vitalité and Revive strongly activate the *glp1* gene to the tune of 348 and 293 percent respectively (**Table 5**). These results suggest that Vitalité and Revive could be an essential part of a proactive wellness regimen focused on supporting a healthy weight.

Supporting and maintaining healthy cognitive function is an important part of any proactive wellness system. Consequently, THREE’s products were assessed for their ability to upregulate *bdnf*—a gene involved in maintaining many different processes in brain health. The data, shown in **Table 6**, found that Vitalité activated *bdnf* expression by 840 percent, Collagène by 570%, and Revive by 430%. These results were surprising and are likely attributable to the large amounts of polyphenols in each of these products. This data supports that these products could help to support brain health through a *bdnf* mechanism of action.

Table 7 shows data on the ability of THREE’s products to regulate the interleukin-6 (*il6*) gene. Downregulation of *il6* is desired in the maintenance of a healthy inflammatory status in the body. Éternel downregulated *il6* by an impressive 1250 percent while Revive and Vitalité were mild down



regulators.

Regarding regulation of the *sirt1* gene, it was found that Revíve increased expression of this gene by 164% (table not shown). Based on the data, Revíve, in addition to maintaining a healthy inflammatory status in the body, might also be able to maintain healthy cellular function, support natural energy production and promote brain health.

Conclusion:

Nutritional supplements help maintain healthy epigenetic function in the body. They represent an exciting new frontier in helping the body live proactively. This study found that THREE's Vitalité, Revíve, Éternel, and Collagène were able to up regulate or down regulate several important genes *in-vitro* related to maintaining energy production, supporting a healthy inflammatory status, maintaining the antioxidant system, supporting healthy blood sugar and blood insulin levels, and promoting cognitive function. From this study we can conclude that THREE's products show significant efficacy in both up and down regulating genes vital to human health. Future studies include applying this same *in-vitro* methodology to human *in-vivo* applications.