



Neutrophil-lymphocyte ratio and update conclusions from a 2-year double-blind rct testing fermented papaya preparation in healthy middle-aged/elderly subjects

Aldo Lorenzetti¹, Antonio Ayala², Fang He³, Cristiana Aperio¹, Leila Haghshenas¹, Maki Osato⁴, Umberto Solimene⁵⁺, Saida Rasulova¹, Francesco Marotta^{1*}

¹ReGenera R&D International for Aging Intervention, Milano, Italy; ²Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of Seville, Spain; ³West China School of Public Health, Dept of Nutrition, Sichuan University, Chengdu, P.R. China. ⁴Redox Molecular Biology Laboratory, ORI Research Institute, Gifu, Japan; ⁵WHO-collaborating Center for Traditional Medicine & Biotechnology, University of Milano, Milano, Italy.

*Corresponding Authors: Francesco Marotta, ReGenera R&D International for Aging Intervention, Milano, Italy

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ABSTRACT

Background: This study represents the second part of a 2-year interventional study in middle-aged /elderly healthy subjects treated either with an antioxidant supplementation (200mg trans-resveratrol, 100mg Centella asiatica extract, 80mg ubiquinol, 50mg epigallo-cathechin-gallate, 20 mg anthocyanidins, 5mg zinc, and 200 IU vitamin E) or with a proprietary fermented papaya preparation (FPP). The choice of the fermented papaya preparation was based on over 12 years of experimental and clinical data proving its effectiveness on redox and immune regulation. The aim of this study was to complete a 24-month investigation of the potential beneficial effect of this functional food on key age-related immune system parameters.

Methods: Study population: From a total of 106 subjects, we analyzed data collected from ninety-eight clinically stable, healthy community dwelling males and females aged 58 to 76 years. The investigation followed a double-blind methodology involving two groups, A and B. Group A received FPP® 4.5 grams, one sachet twice a day, coupled with a placebo capsule in the morning. Meanwhile, Group B was administered a papaya-flavored sachet twice a day, with an additional antioxidant mixture capsule in the morning, referred to as the AA group. Morning blood samples were collected and tested for: Asymmetric dimethylarginine (ADMA), apoptosis of PBMCs and blood including Neutrophil-

Lymphocyte Ratio (NLR) after 12 months, i.e. corresponding to 24 months of the 2-year study (Visit VI of the initial protocol).

Results: As compared to the first preliminary published year of the study, this further 12-month study enabled a more comprehensive analysis with a significant increase of analyzed overweight (≥ 25 and ≤ 29.9 kg/m²) subjects (58 vs 31, $P < 0.05$). On the 24-month mark, AA-group showed a significant increase of NLR ($P < 0.05$ vs entry level and FPP group), whereas FPP-group maintained a stable value. Urinary 8-OHdG values maintained, as in the preliminary study, a wide dispersion of data but both treatments equally reduced its value in over 27BMI/r over 70y old subjects, which as taken separately, had significantly elevated values ($p < 0.05$ vs younger subjects). ADMA and frequency of apoptotic cells values were beneficially reduced only by FPP supplementation throughout all 24 months of the study ($P < 0.05$ vs baseline and vs AA supplementation). A significant correlation between NLR and Apoptosis of PBMCs appeared in FPP-treated subjects and this was more pronounced in subjects with > 27 BMI or over 70 years of age.

Conclusions: The novelty of the present work was the finding that FPP represents a substantial pharma-grade, non-GMO technological advancement over uncontrolled traditional plant extracts. This functional food affects oxi-inflammatory aging phenotype and NLR, a marker worth great attention for its preventive interventional potential in middle-age/elderly communities health care.

Key words: Fermented Papaya Preparation, NLR, ADMA, apoptosis, antioxidants, SARS-Cov19

Fermented Papaya Preparation (FPP-ORI) vs Antioxidants mix in middle-age/elderly people

98 SUBJECTS, DIVIDED IN 2 GROUPS OF 49 EACH
FOLLOW UP OF 24 MONTHS



FPP

VERSUS



Mixed Antioxidants

NLR	24mo. improvement		NO improvement
Ur. 8OHdG/ ($> 72y/BMI > 27$)	improved	±	improved
ADMA	improvement		NO improvement
Annexin V	improvement		NO improvement

Conclusions.. Unlike antioxidants mixture, FPP decreased NLR, ADMA and Apoptosis, all relevant markers of immune system, vascular and systemic aging.

INTRODUCTION

For the last 13 years, a proprietary functional food known as fermented papaya preparation (FPP[®], Osato Research Institute, Gifu, Japan, Patent Number: 6401792) has undergone rigorous validation through numerous experimental and clinical trials, showcasing its enduring immune and redox modulator nutrigenomics effects [1-6]. The Functional Food Center's (FFC) recent consensus has provided a distinct and enhanced definition for evidence-based functional foods, elucidating their impactful bioactivity and the associated biomarkers [7]. This year a brief but stringent position paper further focused on the fundamental regulatory pillars in the field [8]. In the previously published 1-year preliminary report [9] of the present 2-year investigation, we demonstrated that, as compared to the antioxidant-mix supplementation, FPP[®] intervention significantly decreased the asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor and the percentage of apoptotic PBMC, together with a more satisfactory quality of life profile in middle-age/elderly subjects. On the other hand, the dynamics of other variables such as neutrophil lymphocyte ratio (NLR) values, no matter the treatment group, did not reach substantial differences. A recent study by the renowned inChianti group has found that NLR emerges as a superior predictor of healthy aging compared to the isolated assessment of neutrophil or lymphocyte counts. This observation adds valuable insights to the understanding of aging biomarkers [10]. Moreover, it appeared that NLR variations were associated with lifestyle and its surrounding environmental abnormalities, undergoing diseases and the status of immune system [11]. This work corroborated and expanded the NLR-survival rate in non-cancer subjects already shown in 2016 [12]. In the second year of the study, besides monitoring the stability of the results previously achieved, we managed to pool all 98 patients' data thus enabling a thorough and updated analysis of whole study protocol parameters. Specifically,

comprehensive data compilation on NLR could be undertaken, given that elevated values are indicative of unfavorable outcomes in various contexts, including chronic inflammatory conditions, acute infectious diseases, monitoring severity in SARS-COVID-19, and assessing injury induced by environmentally retrievable toxins [13-19]. Given the promising first-year beneficial effect of FPP supplementation in a number of crucial redox parameters and apoptosis regulation, the present study intended to confirm the stability of that data along a further year of observation to study the profile of NLR and its possible relationships with the other variables.

MATERIALS AND METHODS

The study design, conducted in compliance with the Declaration of Helsinki guidelines, was approved by a joint ethical committee of the ReGenera Research Association in January, 19, 2020.

Study population: As stated in the initial section of our inclusion criteria, participants were required to be clinically stable, healthy, community-dwelling individuals of both genders, testing negative for Covid-SARS2, and with a BMI within the range of 23.9 to 32.5 kg/m². Mild dietary-controllable dyslipidemia and borderline hyperglycemia were not grounds for exclusion, provided that participants did not require pharmacological treatment for these conditions. Exclusion criteria were BMI \geq 36, past tumors, major hepato-gastrointestinal, neuropsychiatric, renal or endocrinological disorders, and relevant lung or cardiovascular events in the last 6 months. Antibiotic use as well as fish oils, vitamins, probiotics, nutraceuticals, nicotine/caffeine dependence, or high drinking habit (4+ alcohol-containing beverages on any day or 5+ alcohol-containing beverages per week for women and 7 or more alcohol-containing beverages per week for men), strenuous physical activity in the past 4 weeks or ongoing were regarded as exclusion factors. [9]. From the original start of the study, one year earlier,

both supplementations did not stop. This second year of the study, analyzed 98 subjects, (8 of 106 lost at follow up or dropped out for personal reasons or trauma), all clinically stable, healthy community dwelling males and females (m/f: 42/56) aged 58 to 77 years, Due to the extensive duration of the study, participants' engagement in regular daily activities was monitored using a comparable watch-pedometer application, aiming to maintain an average of 6000 steps/day without specific performance targets.

Design of the study: During the second year of the study, the original double-blind protocol was consistently applied, assessing two distinct treatments. The FPP® group (group A) received FPP® 4.5g, 1 sachet twice a day, with an additional placebo capsule (vegan cellulose) before breakfast. Meanwhile, the AA group (group B) was administered a fruit-flavored 4.5g, 1 sachet twice a day, with the morning dose associated with a capsule containing a comprehensive antioxidant mixture (200mg trans-resveratrol, 100mg Centella asiatica extract, 80mg ubiquinol, 50mg epigallo-cathechin-gallate, 20 mg anthocyanidins, 5mg zinc, and 200 IU vitamin E). This formulation was done by the researchers in a compounding pharmacy by putting together ingredients and dosages much popular in the market. Planned blood tests were set as follows: Visit V at 18 months (only clinical assessments) and Visit VI (final clinical and biochemical assessment) at 24 months.

Methods: Morning blood samples were collected from patients after overnight fasting. In addition to routine clinical laboratory tests, performed blindly, the following parameters were tested: Neutrophil-lymphocyte ratio (NLR), an indicator of systemic inflammation [20] was measured by dividing the number of neutrophils by the one of lymphocytes. As in the previous preliminary study, the following tests were also carried out: Ultra sensitivity C-reactive Protein (by a highly sensitive ELISA with a

lower detection limit of 0.01 mg/L, serum ADMA level by a commercially available enzyme-linked immunosorbent assay (ELISA) kit for ADMA (DLD Diagnostika GmbH, Hamburg, Germany), yielding a sensitivity 0.01 $\mu\text{mol/L}$ and a the inter-assay coefficients of variation of less than 3% [21], urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8OHdG) concentration adjusted for urinary creatinine values [21] by a competitive assay (Oxis Health Products, Inc.). In particular, spot urine samples were collected by subjects in sterile boxes upon awakening (7.00-7.30), before consuming any water or beverages and then taken to the laboratory by 9.00am where they were stored at -70 until the date of assay. Sample aliquots were tested as above for the quantification of 8-OHdG (ng/mL). The urinary creatinine concentration (mg/mL) was quantified by using an ELISA assay kit (Afinion-kit ACR, Italy). The creatinine-adjusted 8-OHdG concentrations (ng/mg) were used for the analysis. To normalize the 8-OHdG concentration distribution, the Box-Cox transformation was used. Univariate associations between subjects' characteristics (age, gender, BMI, fruit, and vegetable consumption) and 8-OHdG concentration levels were evaluated with a linear mixed-effects regression model for repeated measurements.

Annexin V staining was carried out using anti-annexin V antibody (eBioscience) in annexin V binding buffer kept for 15 min. DAPI staining served to exclude the presence of dead cells and the apoptosis frequency was calculated by using BD LSRFortessa (BD Biosciences, San Jose, CA, USA). Apoptosis of peripheral—Blood Mononuclear Cells (PBMCs, isolated from the whole blood by Ficoll-Paque gradient centrifugation. Profiles of each population were analyzed by Cytomics FC 500 (Beckman Coulter).

Statistical analysis: All tests were performed by SPSS version 22 (IBM Inc., Armonik, NY, USA). The normality of the data was checked and confirmed by the Shapiro-Wilk method. Results are presented with means \pm standard

deviations (SD) for continuous variables. We also used the baseline value of FPP® or antioxidant supplementation, as assessed at the start of the study 24 months earlier, to analyze the time-course changes. Differences between the two study groups were calculated by t-test and Chi²-test while a clear-cut alpha level of 0.05 was set for all analyses.

RESULTS

Forty subjects had normal body mass index (18.5–24.9 kg/m²), and fifty-eight were classified as overweight (≥25

and ≤29.9 kg/m²). As compared to the first year of the study, these data showed a significant increase of overweight (≥25 and ≤29.9 kg/m²) subjects (58 vs 31, *P*<0.05). No significant weight change was noted at the end of the study.

NLR values were comparable between the two treatment groups till the 11th month observation. During this observation period, a notable increasing trend was observed in the AA group, culminating in a statistically significant difference at the 24-month control mark (Figure 1, *P*<0.05 vs entry level and FPP group).

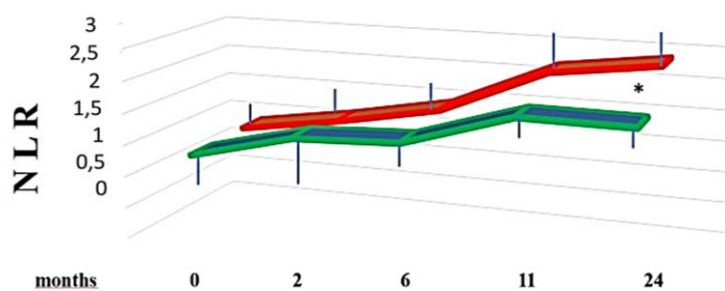


Figure 1. Time-course profile of NLR in both treatments’ groups, AA group (red stripe) and FPP-group (green stripe). * *P*<0.05.

Urinary 8-OHdG values exhibited the previously observed extensive variability between the two groups at baseline (data presented in the previous publication). However, when analyzing these values in subjects aged over 70 or with a BMI exceeding 27, urinary 8-OHdG values were

significantly elevated compared to the overall entry level (*P*<0.05). Both supplementations’ regimes equally improved this variable (Fig 2, *P*<0.05) with FPP® effect started being significant already at 6months till the end of the study (24 weeks).

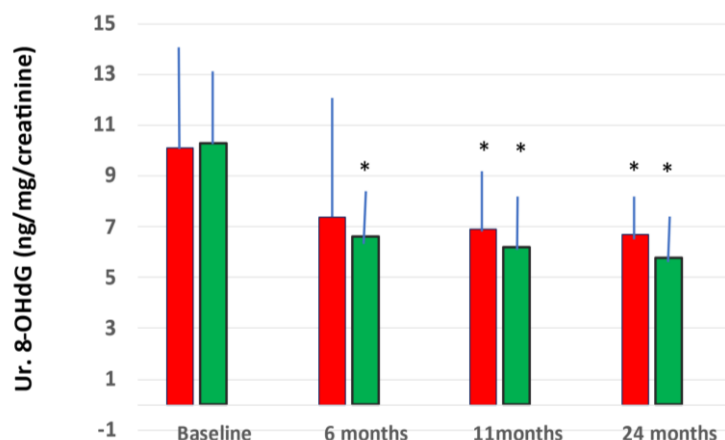


Figure 2. Time-course effect of AA supplemented group (red bars) and FPP-supplemented group (green bars) on the profile of urinary 8OHdG level in the cohort of participants older than 70 years or with BMI >27. Both supplementations enabled a significant and comparable decrease of this parameter as compared to entry level (*P*<0.05).

In contrast, ADMA values remained unaffected by AA supplementation (Fig. 3), while FPP administration exhibited a notable and significant decrease, observed

from the 6-month checkpoint onward ($P < 0.05$ compared to baseline and AA supplementation).

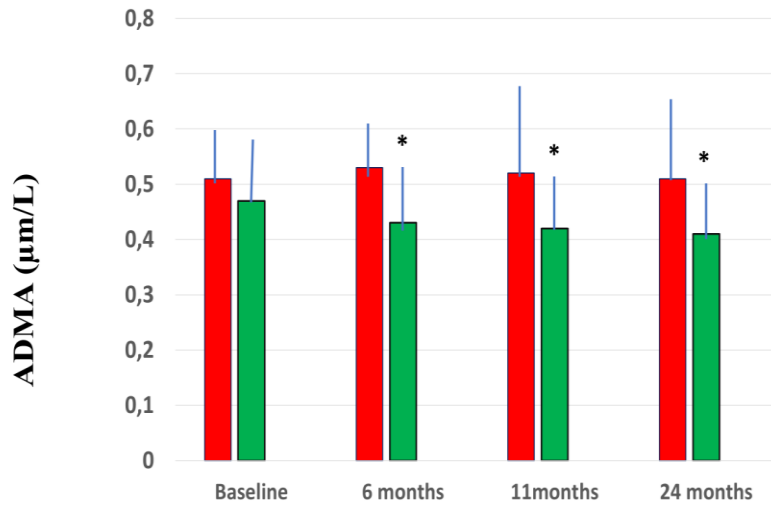


Figure 3. Time-course changes of plasma ADMA level in AA supplemented group (red bars) and in FPP-treated group (green bars). Differently from AA treatment, FPP[®] supplementation yielded a significant decrement till to 24 months control ($*P < 0.05$ vs baseline and AA group).

At the study's onset, there was no notable difference in the frequency of apoptotic cells in PBMCs between the two treatment groups (Fig. 4). Following the 6th month of the trial, the AA group exhibited a gradual and

significant increase ($P < 0.05$ compared to baseline). In contrast, the FPP[®]-administered group showed only a slight, non-significant trend increase while maintaining an overall stable lower percentage ($P < 0.05$ compared to AA group values observed at 6, 11, and 24 months).

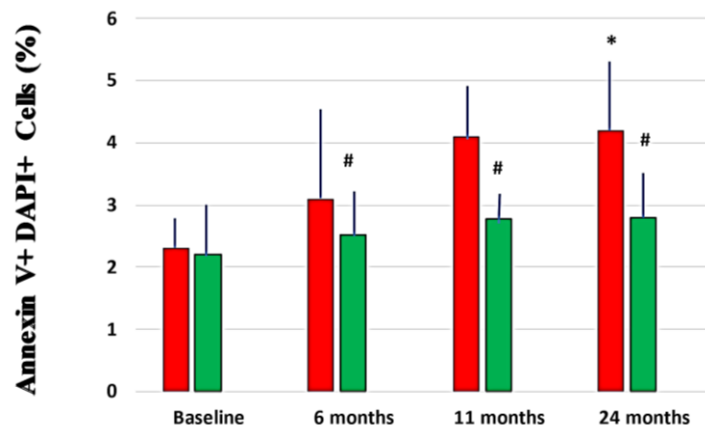


Figure 4. Frequency of apoptotic cells (Annexin V+ DAPI+) at baseline, 6, 11 and 24 months. While this parameter remained fairly stable throughout the 24 month follow up in the FPP[®] group (green bars) ($\#P < 0.05$ vs AA group), , In the AA group (red bars), a notably steep increase was observed at 6 months, and more prominently at 11 and 24 months, reaching statistical significance ($P < 0.01$ compared to baseline).

Table 1 shows the level of statistical significance when correlating the values of NLR and Apoptosis of PBMCs throughout the study period. It appeared that, at 11

months and 24 months, the two parameters were highly correlated, and this was more pronounced in those participants older than 70 years or with BMI >27.

Table 1. Correlation between NLR and Apoptosis of PBMCs in FPP-supplemented group

Elements of correlation	2 weeks	6 weeks	11 weeks	24 weeks
NLR Apoptosis (PBMCs)	r = 0.545 ns	r = 0.524 ns	r = 0.759* *P<0.05 vs baseline	r = 0.772* *P< 0.05 vs baseline
participants older than 70 years or with BMI >27				
NLR Apoptosis (PBMCs)	r = 0.443 ns	r = 0.546 ns	r = 0.791* *P<0.005 vs baseline	r = 0.813** **P<0.005 vs baseline

No correlation appeared between NLR and hCRP, Ur-8OHdG or ADMA at any of the time-check, irrespective of the gender (data not shown). No correlation appeared when analyzing AA group separately, irrespective of age and BMI, ns: not statistically significant.

DISCUSSION

The initial broad range of data observed during the urinary 8-OHdG level assessments in the first year of the study did not allow for definitive conclusions regarding the impact of nutraceutical interventions. This observation persisted until the conclusion of the second-year investigation (data not presented). This finding is not surprising, when considering the disappointing or modest results reported in the past by using vitamin C or E [24-25] and where also the role of diet [26] was not specifically ruled out. For instance, dietary regimens prescribed for chronic benign diseases have indirectly shown to act as cancer protectors [27]. Moreover, such studies, to optimize the homogeneity of the diet, would need a detailed food bioactive components and probiotic

content analysis [28-31]. However, the extended duration of the study confirmed the stability of the results observed during the initial year, persisting until the 24-month observation. . This is significant when considering the increased number of recruited overweight patients (58 vs 31, P<0.05) and aged subjects. This data is in agreement with the observation of drastic improvement of this plasma and urinary parameter after weight loss surgery [32]. NLR can be regarded as a correlated marker of oxi-inflammatory imbalance, associated with cardiovascular disease risk, and serves as a prognostic factor for atherosclerosis, aging, overall mortality, cancer progression, and rheumatoid arthritis [33-39]. This has also been reported in advanced chronic gastritis by Helicobacter Pylori [40]. Nevertheless, even though none of our subjects reported gastric symptoms, considering the susceptibility of the aged population group to oxidative damage [41], we cannot exclude the potential presence of gastric atrophy with various immune-inflammatory mucosal rearrangements [42]. The anticipated positive effects of FPP [43] at this specific

level are evident, as demonstrated in a clinical study [44]. Interestingly, NLR and ADMA, although not yielding a statistical correlation between each other, showed a similar higher level and, unlike the AA group, both parameters significantly responded to FPP[®] at 11 and 24 monthss. Exercising due caution due to the severity of the disease, a recent study conducted on hospitalized SARS-CoV-2 patients revealed an association between mortality and concurrent abnormalities in NLR and ADMA [45]. This supports the prognostic value of ADMA assessments in these patients, both during hospitalization and throughout the post-COVID syndrome period, which is still not fully understood [46-50]. Although none of our patients had diabetes, we did not conduct HOMA testing. In this expanded group of overweight individuals, HOMA testing might have revealed insulin resistance in some cases. Existing literature clearly highlights that NLR abnormality is associated with this condition [51] and is responsive to antioxidant treatment [52]. Although our study did not address cerebrovascular pathophysiology, a recent review from Framingham Heart Study has reported that subjects with higher NLR are at a greater risk of later dementia during a 5.9-year follow-up period [53]. This area is understandably of extremely complex metabolic interrelations, either environmentally (exotoxins) [54, 55] or internally (endotoxins) [56].

In this nutritional interventional setting, Belpomme's group [57], by using FPP[®], has demonstrated a statistically significant clinical and redox benefit together with an increase in intracerebral tissue pulse metric index in the temporal lobes through ultrasonic brain tomosphygmography and whose long-term relevance warrants follow up studies. Amid the accumulating evidence of effective functional foods

influencing brain physiology [58-64], this signifies a step beyond the raised caution associated with intrinsic methodological variables in in vitro analysis [65]. In the quest for a rational science-validated functional food [66] and more controlled selection of bioactive compounds [67], FPP represents a substantial demonstration of pharma-grade, non-GMO technological advancement over traditional carica papaya use [68]. The ongoing pursuit of healthy living inevitably involves impacting a fundamental oxi-inflammatory phenotype to varying degrees [69-71]. In this context, NLR monitoring [72] may be a marker worth great attention [73-75] and preventive interventional potential in middle-aged/elderly communities health care [76-77]. The novelty of the present study resides in a long-term monitoring of key age-related parameters while using a functional food treatment.

CONCLUSION

This groundbreaking study represents the first two-year monitoring of the effects of antioxidants and a novel functional food (FPP) on key redox parameters, PBMCs apoptosis, and the NLR, with a particular emphasis on the long-term impact. While the antioxidants mixture exerted a significant effect on redox parameters, on top of this, FPP was shown to bring about age/health- and longevity-associated immune system markers. This finding may help substantiate health maintenance strategies.

Abbreviations: NLR: neutrophil-lymphocyte ratio, i.e. the number of neutrophils divided by the number of lymphocytes; ADMA: Asymmetric dimethylarginine; PBMCs: Perypheral Blood Mononuclear Cells; FPP[®]: Fermented Papaya Preparation; BMI: Body Mass Index; 8OHdG: 8-oxo-7,8-dihydro-2'-deoxyguanosine

Contribution: OM, SU discussed the research plan, LA and RS followed the clinical aspects FH and AC discussed and evaluated nutritional aspects, AA and US discussed and evaluated the biochemistry.

Conflict of interest: Authors declare no personal or institutional conflict of interest.

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