

## Oleic Acid, Deglycosylated Vitamin D-Binding Protein, Nitric Oxide: A Molecular Triad Made Lethal to Cancer

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**Abstract.** *Background:* Oleic Acid (OA) has been shown to have anticancer properties mediated by interaction with proteins such as  $\alpha$ -lactalbumin and lactoferrins. Therefore, we synthesized complexes of OA and Gc protein-derived macrophage activating factor (GcMAF) that inhibits per se cancer cell proliferation and metastatic potential. We hypothesised that OA-GcMAF complexes could exploit the anticancer properties of both OA and GcMAF in a synergistic manner. We postulated that the stimulating effects of GcMAF on macrophages might lead to release of nitric oxide (NO). *Patients and Methods:* Patients with advanced cancer were treated at the Immuno Biotech Treatment Centre with OA-GcMAF-based integrative immunotherapy in combination with a low-carbohydrate, high-protein diet, fermented milk products containing naturally-produced GcMAF, Vitamin D3, omega-3 fatty acids and low-dose acetylsalicylic acid. *Results:* Measuring the tumour by ultrasonographic techniques, we observed a decrease of tumour volume of about 25%. *Conclusion:* These observations demonstrate that OA, GcMAF and NO can be properly combined and specifically delivered to advanced cancer patients with significant effects on immune system stimulation and tumour volume reduction avoiding harmful side-effects.

It is well-assessed that Oleic Acid (OA), a recognised fundamental component of healthy diets (1), shows anticancer properties (2) that contribute to the increase in

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longevity and reduced risk of mortality and morbidity associated with its consumption (1). Although the precise molecular mechanism responsible for its anticancer properties is not completely understood (2), it appears that OA is involved in intracellular calcium signalling associated with the induction of cancer cell apoptosis.

It has been proposed that the anticancer effects of OA are mediated by its interaction with proteins highly represented in biological fluids such as  $\alpha$ -lactalbumin and lactoferrins. These proteins bind OA to form OA-protein complexes which exhibit highly selective anti-tumour activity *in vitro* and *in vivo* (3). Soon after their identification, these complexes were labelled as HAMLET an acronym that stands for “human  $\alpha$ -lactalbumin made lethal to tumour cells”, even though further studies demonstrated that  $\alpha$ -lactalbumin is not the sole protein forming such complexes. Thus, other proteins, forming complexes with OA, exhibit identical anticancer properties (4). It is now accepted that these OA-protein complexes destroy tumour cells with high selectivity and with no evidence of toxicity for normal tissues, a key feature in the quest for anticancer treatments devoid of toxic side effects.

Study of the structural characteristics of such anticancer OA-protein complexes demonstrated that a common molecular feature is the tendency toward OA-induced protein oligomerization. Since OA-induced oligomerization has been reported for a number of proteins in addition to those initially identified in HAMLET, it was hypothesised that this phenomenon may be inherent to many proteins (5).

Some of the proteins forming OA-protein anticancer complexes such as  $\alpha$ -lactalbumin and lactoferrins are highly represented in milk. These compounds are known to exert powerful stimulatory effects on the immune system (6). These findings provide additional acceptance to the hypothesis that the immune system is directly involved in the overall anticancer effects of OA-protein complexes.

In addition to  $\alpha$ -lactalbumin and lactoferrins, another immunostimulatory protein highly represented in milk, as well as in colostrum and blood, is the vitamin D binding protein. This is the precursor of a very potent macrophage activating factor that derives from its selective deglycosylation. Since vitamin D binding protein is also termed Gc-globulin, this macrophage-activating factor is known as GcMAF (Gc-globulin-derived Macrophage Activating Factor) (for a review on vitamin D binding protein and GcMAF, see Reference 7).

The potent immunotherapeutic effects of GcMAF in human tumours have been demonstrated since 2007 in a variety of cancers ranging from the most common breast and prostate cancers to the less frequent oligodendroglioma (8-14).

Therefore, considering that GcMAF binds OA in a manner identical to that of  $\alpha$ -lactalbumin or lactoferrins (15), we hypothesised that OA-GcMAF complexes were endowed with anticancer activity greater than that of OA or GcMAF alone when combining the anticancer properties of GcMAF with those of OA-protein complexes. Since OA-protein complexes and GcMAF show no evidence of toxicity (4, 16), an OA-GcMAF complex was used as part of an integrative immunotherapeutic approach to advanced cancers in the context of the so-called compassionate approach (14).

In the present study we hypothesise that the dramatic effects of OA-GcMAF in cancer patients recently reported (14) are mediated by the production of nitric oxide (NO) by activated macrophages. This identifies a triad of molecular elements, OA, GcMAF and NO, each one endowed with individual anticancer properties, that demonstrate synergistic effects and might open the way to a much improved cancer treatment strategy devoid of harmful side effects.

## Patients and Methods

At the Immuno Biotech Treatment Centre, patients with advanced cancer are currently being treated with OA-GcMAF-based integrative immunotherapy. OA-GcMAF complexes are used in combination with the following approaches: (i) A very low carbohydrate, high protein, equicaloric diet that is known to slow tumour growth and prevent cancer initiation (17), (ii) Fermented milk products containing naturally produced GcMAF, (iii) High Vitamin D3 supplementation (18), (iv) Low-dose acetylsalicylic acid (19) and (v) Omega-3 fatty acid supplementation (20).

These approaches, aimed at strengthening the immune system and reducing tumour growth, are considered complementary and not alternative to other anti-neoplastic therapeutic procedures that the patients and their caring health professional may want to take into consideration.

*Preparation of OA-GcMAF complexes.* OA-GcMAF complexes (GOleic) were prepared in-house at Immuno Biotech Ltd, with a proprietary procedure. Briefly, GcMAF was purified according to the procedure previously described (10). Vitamin D-binding protein was isolated from purified human serum obtained from the American Red

Cross (2025 E St NW Washington, DC, USA), using either 25-hydroxyvitamin D3-Sepharose high affinity chromatography or actin-agarose affinity chromatography. The bound material was eluted and then further processed by incubation with three immobilized enzymes as described (16). The resulting GcMAF was filter sterilized. The protein content and concentration was assayed using standard Bradford protein assay methods (21). Purity was assessed by SDS-PAGE and Western Blot analysis performed after each step of the preparation procedure; only one band of the expected molecular weight was visible (22). At the end of the production process, GcMAF was checked for sterility in-house and externally by independent laboratories. Its safety and biological activity were tested in human monocytes, human breast cancer cells and chick embryo chorionallantoic membrane (15, 23, 24). Highly purified OA (molecular weight, 282.46; molecular formula, C18 H34 O2; Acros Organics, Geel, Belgium) was complexed with GcMAF in accordance with the molecular structures and modelling already described (15). The optimal conditions for the preparation of the complexes were established according to described principles (25).

Due to the complexing of the protein with the fatty acid hydrophobic moiety and in view of the well-known properties of OA as an absorption enhancer, OA-GcMAF complexes could be administered sublingually (26), as an aerosol with a common nebuliser (27), as suppositories (28) or transdermally (29).

*Assessment of OA-GcMAF-induced immunotherapeutic effects.* Macrophage activation *in vivo* was assessed by monitoring the patients' blood pressure and the splenic blood flow before and after OA-GcMAF administration. Thus, it is well known that activated macrophages release NO, a compound that causes vasodilatation and seems to be responsible for some of the anticancer properties of activated macrophages (30, 31). The administration of OA-GcMAF (440-880 ng dissolved in 5 ml saline) with a nebuliser, resulted in rapid decrease of blood pressure with the effects clearly appreciable 1 min after the end of the nebulisation. In order to have another assessment of NO production and immune stimulation, the splenic blood flow was monitored with an ultrasound system (MyLab25Gold, Esaote, Genoa, Italy) using the echo-colour-Doppler technique.

*Integrative cancer immunotherapy with OA-GcMAF.* The standard protocol of the Immuno Biotech Treatment Centre is as follows: OA-GcMAF (880 to 2,000 ng according to the patients' needs) is administered daily using the route of administration that is most suitable for each patient e.g., in patients with lung cancer or metastases, administration of OA-GcMAF with a nebuliser is preferred. In patients with liver cancer or metastases, administration through suppositories may be used. In other cases, the intramuscular route, as originally proposed by Yamamoto *et al.* (8), is used.

*Other complementary integrative approaches.* In order to exploit the known anticancer properties of Vitamin D3, and bearing in mind that GcMAF is a component of the vitamin D axis (15), patients are provided with nutritional supplementation of Vitamin D3, 20,000 IU per day (18) monitoring the blood levels of such a vitamin. Patients are taught to drink at least 2 litres of water (or other liquids such as herbal teas) per day.

Patients are instructed to follow a nutritional regime based on the recent observation demonstrating that a low carbohydrate, high protein diet slows tumour growth and prevents cancer initiation (17). In order to favour the compliance to this type of diet, patients are provided with food containing only 2% of carbohydrates and a

relatively high protein content (Le Gamberi Foods, Forlì, Italy). In addition, in order to exploit the known anticancer properties of OA contained in extra-virgin olive oil (32), patients are provided with extra-virgin olive oil as a conspicuous portion of their daily fat intake.

Patients' weight and muscle mass are constantly monitored, and patients are taught the strategies to decrease the Prognostic Inflammatory Nutritional Index (PINI) score as well as the strategies to avoid Cancer Anorexia Cachexia Syndrome (CACS) according to Fabris *et al.* (33). The supplementation of aminoacids (Master Aminoacid Pattern, dr. reinwald healthcare gmbh, Schwarzenbruck, Germany) is intended for this scope (34). Also the administration of low-dose acetylsalicylic acid and of extra-virgin olive oil are intended to reduce systemic inflammation and, therefore, to reduce the PINI score (35).

In order to exploit the well-known immune stimulating and anticancer effects of probiotic fermented milk products (36), patients are provided with a probiotic fermented milk product containing colostrum and microorganisms known to produce natural GcMAF from milk and colostrum Gc-globulin during the fermentation process (Bravo Probiotic, Les Alpes, Wellington, NZ). Finally, considering the well assessed role of low-dose acetylsalicylic acid in cancer prevention (37), patients are provided with 100 mg of such an active principle per day.

## Results

It is well-assessed that OA-GcMAF stimulates macrophages (15) which in turn release NO (30). Considering the impressive effects of NO in pre-clinical models of cancer where it slows tumour growth and enhances the efficacy of both chemotherapy and radiotherapy (38), as a first step we sought to determine whether OA-GcMAF stimulated the release of NO from macrophages *in vivo*. To this end, we chose to study the variation of systolic and diastolic blood pressure and of the splenic blood flow, following administration of OA-GcMAF (880 ng in 5 ml saline) by nebulisation. According to our hypothesis, OA-GcMAF would stimulate alveolar macrophages that in turn would produce NO, thus lowering blood pressure. OA-GcMAF would then be absorbed into the bloodstream and it would eventually stimulate macrophages in other areas of the body. Considering the huge amount of macrophages residing in the spleen (39), we would expect a massive release of NO by activated macrophages in this organ, with a concomitant increase of blood flow to be measured easily by echo-colour-Doppler.

A subject with mild immunodeficiency due to multiple thyroid nodules (test subject # 1; Figures 1 and 2), self-administered OA-GcMAF (880 ng in 5 ml saline) by nebulisation for about 5 min. About 1 min after the end of nebulisation, blood pressure, taken while the subject was comfortably seated, decreased from 124/85 to 115/74. A qualitatively similar effect was observed in the majority of the subjects who were administered OA-GcMAF by nebulisation, and the average decrease was in the order of 10 mm Hg. We interpreted these results as indirect evidence of NO production by OA-GcMAF-activated alveolar macrophages.

In fact, it is well-assessed that NO decreases blood pressure in humans as well as in experimental animal systems (40, 41).

Figure 3, shows the ultrasonographic appearance of the spleen of test subject #1 prior to nebulisation; spleen morphology, size and ultrasonographic structure appear normal. The splenic blood flow could be appreciated in correspondence of the hilum of the spleen. Little or no signal could be observed in the parenchyma of the organ. Figure 4 shows the splenic blood flow 5 min (panel A) and 1 h after nebulisation (panel B). The increase in parenchymal blood flow is impressive. We interpreted this increase in parenchymal blood flow as a consequence of intra-splenic macrophage activation with the release of NO that in turn was responsible for hilar and peripheral blood vessel dilation. This effect lasted for at least 48 h. Figure 5, depicts the splenic blood flow 24 h (panel A) and 48 h (panel B) after nebulisation. Notice that test subject #1, unlike *bona fide* patients, did not undergo any other treatment, neither GcMAF-related nor associated with the other complementary strategies described above.

The reliability and reproducibility of the method was quickly introduced as part of the general assessment of patients' responsiveness to OA-GcMAF administration. A similar effect, although much slower in its development, was observed in patients where OA-GcMAF was administered by localised subcutaneous injections. Figure 6 shows the increase in splenic blood flow observed after 7 days of treatment in a patient with metastatic breast cancer and retrosternal node involvement, where the OA-GcMAF (1.320 ng/day) was administered by localised subcutaneous injections in the abdominal wall (epigastrium, 880 ng) and the axilla (440 ng).

Activation of alveolar and splenic macrophages by OA-GcMAF administration with consequent release of NO was associated with significant decrease in tumour volume in all cases where the primary tumour, metastases or lymphnodes could be measured by ultrasonographic techniques. On average, we observed a decrease of tumour volume of about 25% in a week (14). Although this reduction may appear dramatic, it is fully consistent with the results reported by Nonaka *et al.* (42) who observed a 97% volume reduction of human hepatocellular carcinoma after 3 weeks of subcutaneous injection with GcMAF. It is also consistent with the results reported for neo-adjuvant chemotherapy (43). These results add support to the hypothesis that OA, GcMAF and NO, molecules endowed with individual anticancer properties targeting different aspects of neoplastic growth, provide an indication for a synergistic effect when administered in such a way to exploit their physiological characteristics.

Among the cases observed at the Immuno Biotech Treatment Centre, we present the cases of two patients, each representative of common cancers, for whom the integrative immunotherapy based on OA, GcMAF and NO, was

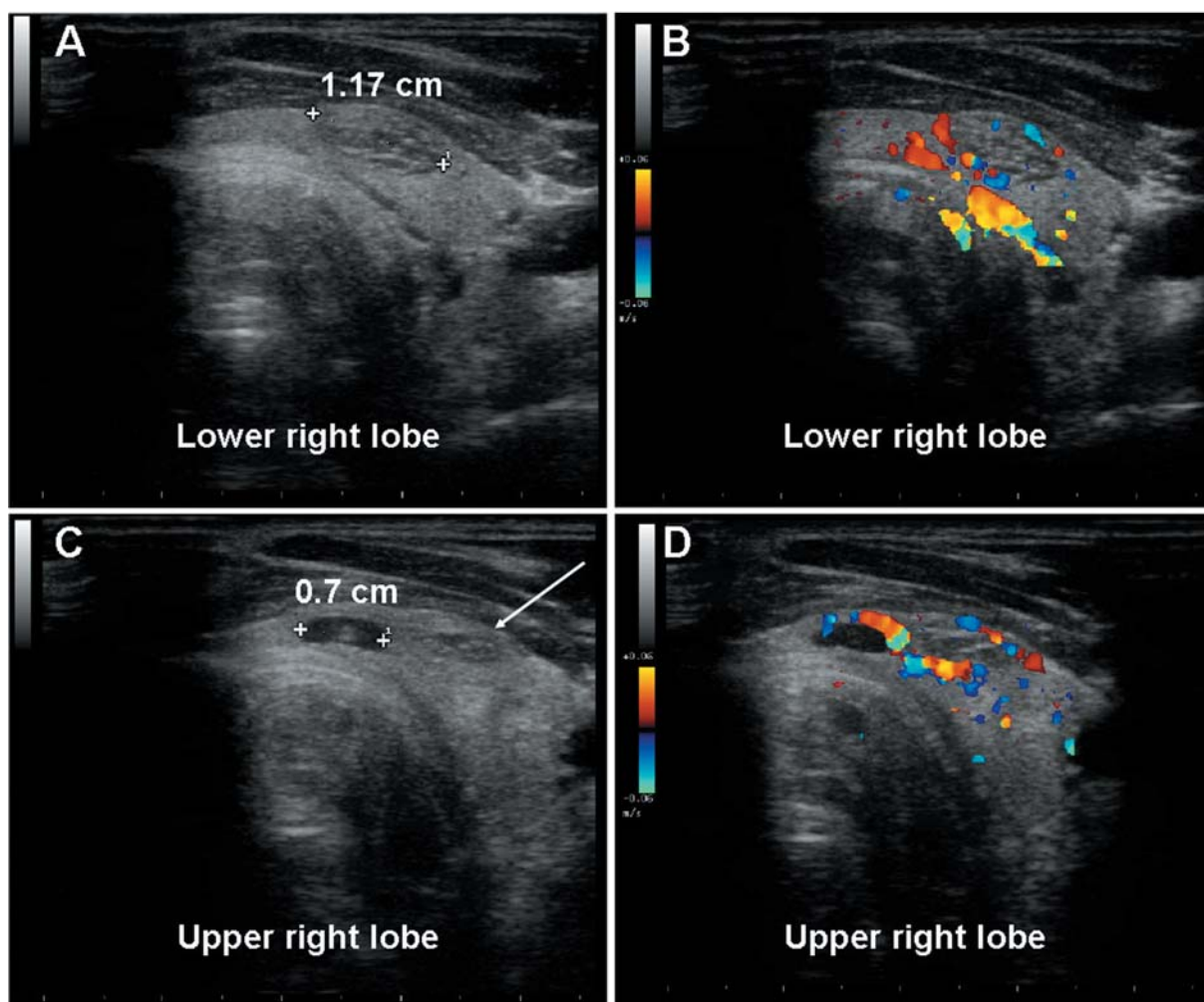


Figure 1. Ultrasonographic findings of test subject #1: thyroid ultrasonography, axial scan of the lower right lobe. A. Lightly-protruding solid nodule of 1.17 cm in the anterior part of the lower lobe. The internal ultrasonographic structure is finely inhomogeneous and hypo-reflecting in comparison with the surrounding normal thyroid tissue. The margins are well-defined. B. Color-Doppler ultrasonography of the same nodule. The blood vessels are located around the solid nodule in a typical “basket-like” appearance. C. A smaller, prevalently liquid (hypo-reflecting), lesion (0.7 cm) with another small solid nodule in the vicinity (white arrow). Inside the hypo-reflecting lesion, an area of protruding solid tissue can be noticed. D. Color-Doppler ultrasonography of the same lesion. The blood vessels are located around the two lesions.

remarkably effective. To our knowledge, this is one of the few examples of actual images of tumour volume reduction following GcMAF immunotherapy. Thus, so far the effectiveness of immunotherapy of cancer with GcMAF has relied upon non-specific markers such as serum  $\alpha$ -N-acetylgalactosaminidase levels (8-10, 12) or anecdotal reports (11, 13).

*Patient 1.* A 56-year-old man was diagnosed with metastatic squamous cell carcinoma, previously treated with several cycles of radiation therapy and chemotherapy. After a period of remission, several new metastases were detected, including one in the soft tissues of the right

abdominal wall. In addition, the patient showed difficulty in breathing, probably because of bronchi-obstructing metastases that prevented ventilation of his apical right lobe. Because of these symptoms, OA-GcMAF (880 ng/day) was administered with a nebuliser containing 440 ng of the active principle dissolved in 5 ml of saline, and with localised subcutaneous injections (440 ng) in the right abdominal wall in proximity of the subcutaneous lesion indicated by the patient and easily recognisable by ultrasonography. The patient did not report any adverse effects following these ways of administration that had been chosen with his informed consent. Ultrasonography of the lesion in the right abdominal wall was performed to

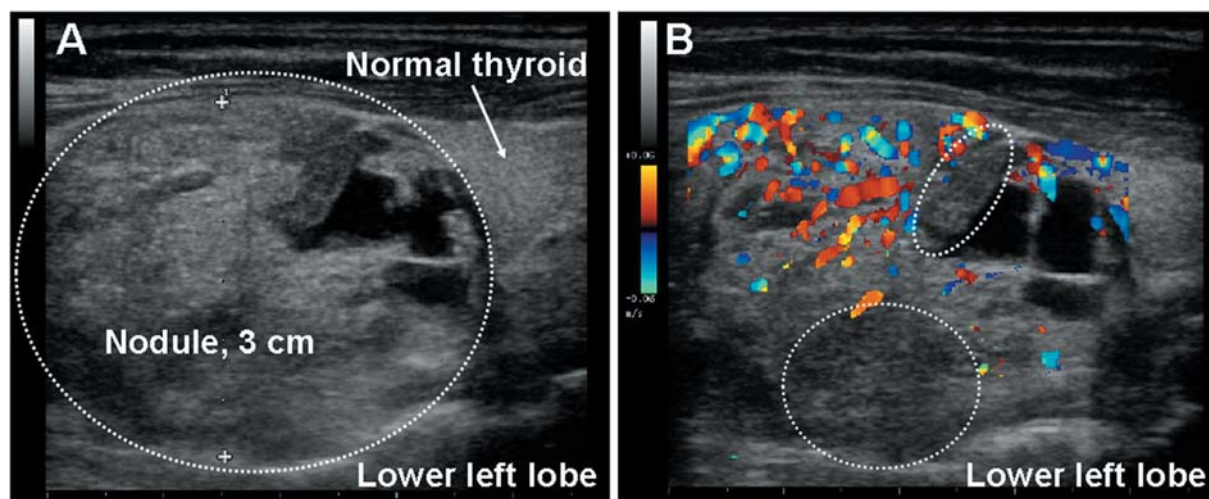


Figure 2. Ultrasonographic findings of test subject #1: thyroid ultrasonography, longitudinal scan of the lower left lobe. A. A massive mixed (solid and cystic) protruding nodule substituting for most of the lobe can be observed. The anterior-posterior diameter is about 3 cm, whereas the longitudinal diameter was greater than the length of the probe (4 cm). The structure is grossly inhomogeneous with cystic areas and hypo-reflecting areas alternated with iso- or hyper-reflecting areas. The margins bordering the residual normal thyroid tissue observable on the right of the figure (white arrow, cranial portion of the left lobe) appear well defined. The hypo-reflecting border might indicate an area of oedema surrounding the nodule. B. Color-Doppler ultrasonography of the same nodule. The blood vessels are located around and inside the nodule. It is worth noticing that few or no blood vessels can be observed in the hypo-reflecting areas (indicated by the dotted white circles) suggesting the presence of metabolically inactive tumour tissue.

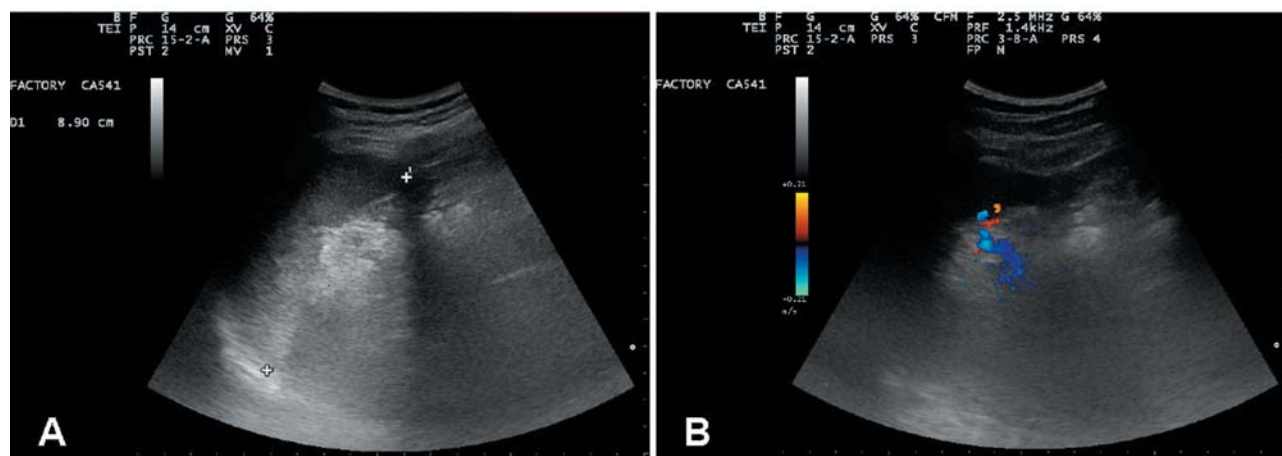


Figure 3. Ultrasonographic appearance of the spleen of test subject #1 before OA-GcMAF administration. Panel A: Morphology, size and ultrasonographic structure of the spleen appear normal. Panel B: Splenic blood flow can be appreciated in correspondence of the hilum of the spleen. Little or no signal could be observed in the parenchyma of the organ.

assess modifications. Bearing in mind that measurements taken on ultrasonographic images may be affected by a number of variables, preliminary evidence appears to indicate that this lesions, taken as representative, showed a reduction of its calculated volume from 0.30 to 0.22 ml (possibly 27%) after 5 days of treatment (Figure 7). The patient reported a subjective improvement of breathing and ventilation in the apical right lobe was improved as well.

*Patient 2.* A 62-year-old woman was diagnosed with extensive breast cancer with widespread axillary lymph node involvement. The patient had elected not to undergo surgery or any other conventional treatment since the initial diagnosis. Because the size of the primary tumour was larger than the probe (4 cm), we opted for measuring the coalescent axillary nodes in order to monitor a response to OA-GcMAF. OA-GcMAF (880 ng/day) was administered with a nebuliser

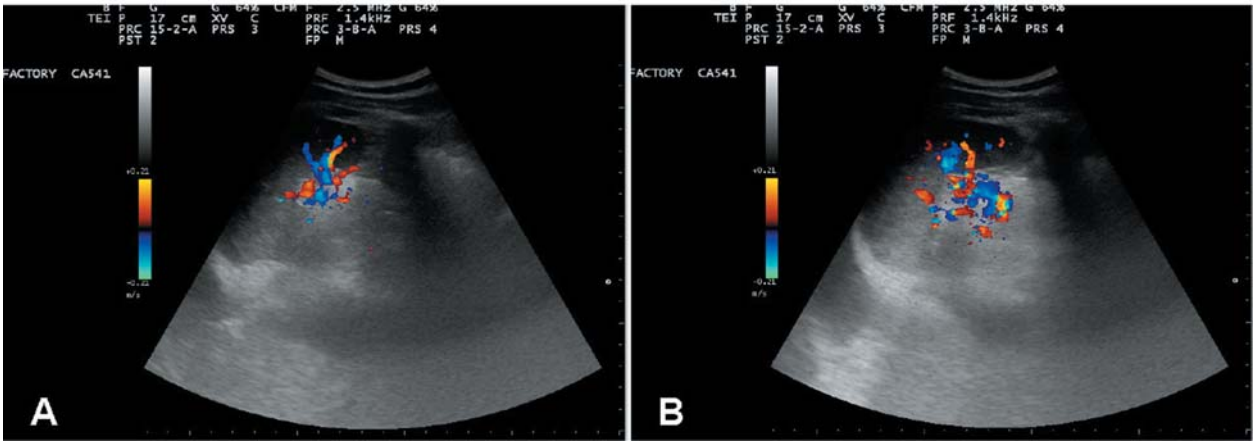


Figure 4. Immediate increase of splenic blood flow following administration of OA-GcMAF by nebulisation in test subject #1. Splenic blood flow was assessed by an echo-colour-Doppler ultrasonographic technique. Panel A: Splenic blood flow 5 min after the end of nebulisation with OA-GcMAF. Panel B: Splenic blood flow 1 h after the end of nebulisation. The increase in parenchymal blood flow is impressive.

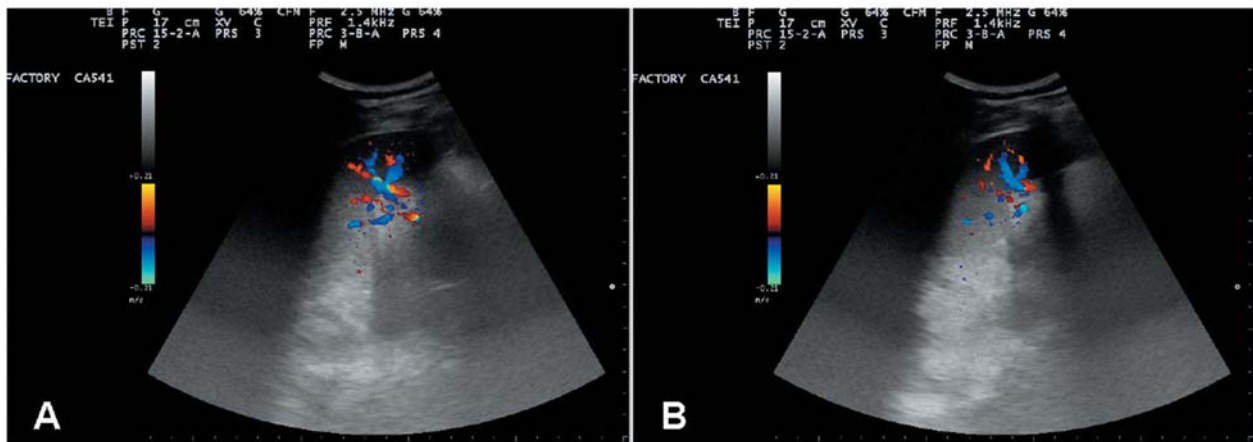


Figure 5. Sustained increase of splenic blood flow following administration of OA-GcMAF by nebulisation in test subject #1. Splenic blood flow was assessed by echo-colour-doppler ultrasonographic technique after administration of OA-GcMAF. Panel A: Splenic blood flow 24 h after the end of nebulisation. Panel B: Splenic blood flow 48 h after the end of nebulisation. The increase in parenchymal blood flow appears to be persistent.

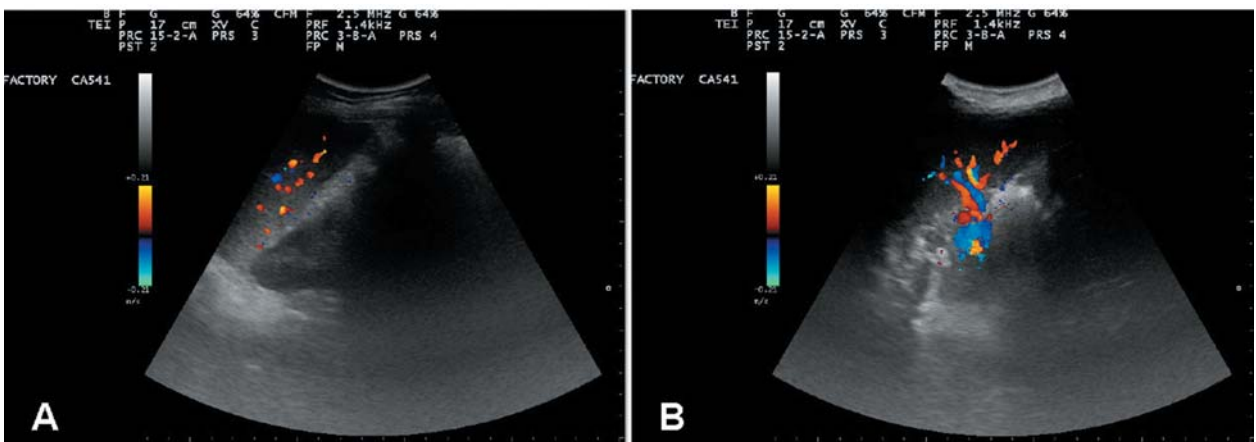


Figure 6. Increase of splenic blood flow following administration of OA-GcMAF by localised subcutaneous injection in a cancer patient. Splenic blood flow was assessed after administration of OA-GcMAF by an echo-colour-Doppler ultrasonographic technique. Panel A: Splenic blood before administration. Panel B: Splenic blood flow after 7 days of administration. The increase in parenchymal blood flow is evident.

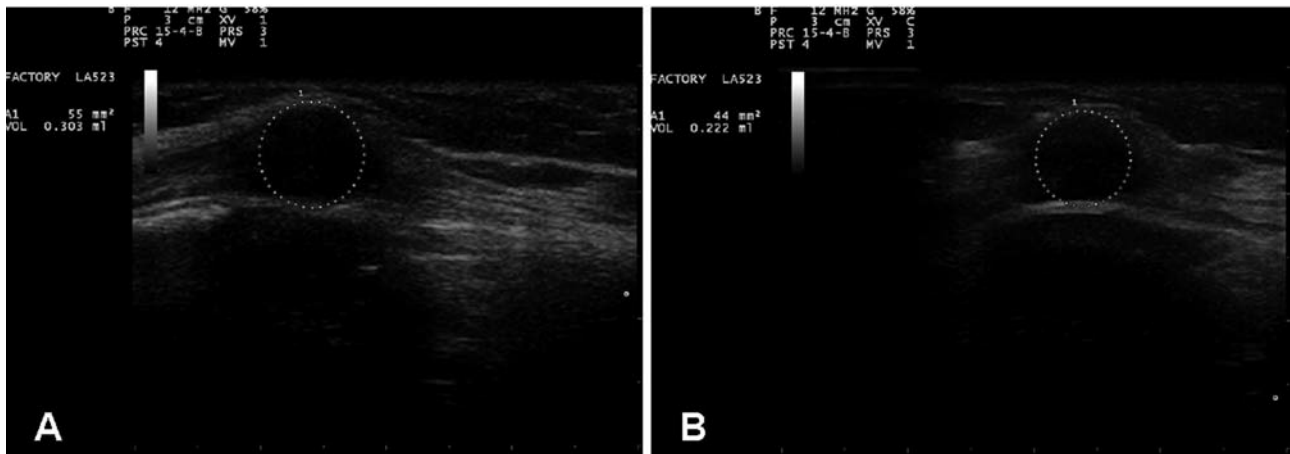


Figure 7. Ultrasonography of soft tissue metastasis before and after OA-GcMAF administration. Patient #1: A 56-year-old man was diagnosed with metastatic squamous cell carcinoma, previously treated with several cycles of radiation therapy and chemotherapy. After a period of remission, several new metastases were detected, including one in the soft tissues of the right abdominal wall. Panel A: Ultrasonographic appearance of the lesion before OA-GcMAF administration. Panel B: Ultrasonographic appearance of the lesion after 4 consecutive days of OA-GcMAF administration. An approximate 27% volume reduction can be appreciated.

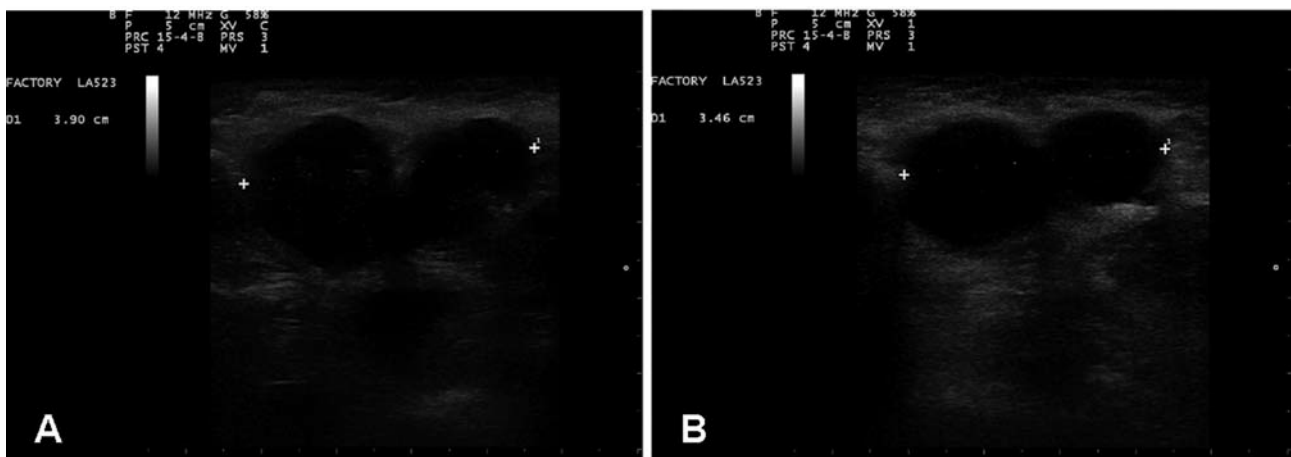


Figure 8. Ultrasonography of neoplastic axillary lymphnodes before and after OA-GcMAF administration. Patient #2: A 62-year-old woman, was diagnosed with extensive breast cancer with widespread axillar lymphnode involvement. Ipsilateral coalescent axillary nodes were measured. After 4 consecutive days of OA-GcMAF treatment the diameter of the two coalescent axillary nodes was reduced from 3.90 (panel A, i.e. before treatment) to 3.46 cm (panel B, i.e. after 4 days of treatment). In terms of calculated theoretical volume, assuming that the lesion was spherical, this corresponds to a reduction of about 28%.

containing 440 ng the active principle dissolved in 5 ml of saline, and with localised subcutaneous injections (440 ng) in the axilla, 1 cm distal to the nodes identified by ultrasonography. The Patient did not report any adverse effects following these ways of administration that had been chosen with her informed consent. Figure 8 shows that after 4 days of treatment the diameter of the two coalescent axillary nodes was reduced from 3.90 to 3.46 cm. In terms of calculated theoretical volume, assuming that the lesion was spherical, this corresponds to a reduction of about 28%.

## Discussion

OA, GcMAF and NO are molecules endowed with multiple biological activities that can be exploited in anticancer treatment strategies. The selective tumour killing effects of OA-protein complexes such as HAMLET, is attributed to OA that, once carried inside the tumour cells by virtue of its association with proteins, activates specific steps in tumour cell death. These include interference with oncogene expression (*Ras*, *erbB-2* and *c-Myc*), and with glycolytic

enzymes (2, 4). In this scenario, the protein serves mostly, if not uniquely, as a vehicle to carry OA inside the tumour cell where the fatty acid can target the cytoplasmic membrane, cytoskeleton, mitochondria, proteasomes, lysosomes and nucleus (4). Thus, a recent study demonstrated that the biological activities of OA- $\alpha$ -lactalbumin complexes reside in the fatty acid. Also, the  $\alpha$ -lactalbumin moiety does not have a tumour-killing effect on its own but merely acts as a solubilising agent for OA (44).

Therefore, the synthesis of OA-GcMAF complexes might represent an advantage over HAMLET since GcMAF, unlike  $\alpha$ -lactalbumin, exerts a direct effect on cancer cells. In particular, we and others demonstrated that GcMAF inhibits cancer cell proliferation and metastatic potential (24, 45). In other words, OA-GcMAF complexes could exploit the anticancer properties of both OA and GcMAF, possibly in a synergistic manner. In this scenario, OA would favour the binding of the complex to the cytoplasmic membrane as already demonstrated (15) and the OA-GcMAF-vitamin D receptor (VDR) multi-molecular complex would be internalised. Once inside the cell, OA and GcMAF could target their respective metabolic/signalling pathways rapidly inducing cancer cell apoptosis.

As a matter of fact, we recently observed that OA-GcMAF complexes are about 200-fold more potent than GcMAF alone in inducing human breast cancer cells apoptosis *in vitro* (Thyer L, *et al.*, personal communication of experimental data presented at the 18<sup>th</sup> International Meeting of the European Society of Gynecological Oncology, Liverpool, U.K., October 2013), possibly because of this synergistic effect.

If this hypothesis is correct it might help explain the direct effects of OA-GcMAF complexes on tumour cells; the distinct stimulatory effects of GcMAF on macrophages might be responsible for the synthesis and release of a third anticancer molecule that is NO. Thus, we demonstrated that the effects of GcMAF are mediated by VDR (15, 46), and VDR activation leads to the synthesis and release of NO by macrophages (47). In our observation, the synthesis and release of NO following OA-GcMAF administration could be inferred by the decrease in blood pressure and increase in splenic blood flow (see "Results" section).

The anticancer effects of NO have been known for many years (30) and it appears that OA and NO share common targets in cancer cells. Thus, NO suppresses cellular respiration and DNA synthesis and induces cancer cell apoptosis by activating the caspase family proteases, a feature in common with OA (2, 48). Most interesting, however, from the point of view of clinical application, are the converging effects of OA and NO on cancer cell glycolytic pathways (49, 2).

Consistent with the hypothesis of glycolysis as a primary target for OA and NO anticancer effects, it was

demonstrated that glucose deprivation sensitized tumour cells to HAMLET-induced cell death (50). Indeed, this observation was at the basis of one of the tenets of the integrative anticancer approach described above, which is the low carbohydrate nutritional plan. In fact, we reasoned that the low carbohydrate diet would not only deprive the cancer cells of their sole source of energy (17) but it would also make them more susceptible to the anticancer effects of OA and NO.

In conclusion, the observations presented in this study, together with the recent results of molecular modelling (15), demonstrate that OA, GcMAF and NO, molecules endowed with individual anticancer properties, can be properly combined and specifically delivered to advanced cancer patients with significant effects on immune system stimulation and tumour volume reduction. In fact, out of the 40 patients who met the Immuno Biotech Treatment Centre acceptance criteria, most with late stage 4 cancers, all showed similar and significant clinical improvements. These observations have the potential for revolutionising the field of cancer treatment since it appears that the proposed anticancer strategies are entirely devoid of any recorded harmful side effects.

### Potential Conflicts of Interest

DN is the CEO of Immuno Biotech, Ltd (the company isolating and purifying the GcMAF protein and producing OA-GcMAF). However, DN had no knowledge of the therapies being used nor of the names of any patients whose data were being analyzed. Neither he, nor any employee of Immuno Biotech, Ltd, had any knowledge of the clinical records or the patients' names used in this study. MR is consultant scientific director of Immuno Biotech, Ltd, and is full professor of molecular biology; he contributed to the described molecular modelling and result interpretation.

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### References

- Martín-Peláez S, Covas MI, Fitó M, Kušar A and Pravst I: Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. *Mol Nutr Food Res* 57(5): 760-771, 2013.
- Carrillo C, Cavia Mdel M and Alonso-Torre SR: Antitumor effect of oleic acid; mechanisms of action: a review. *Nutr Hosp* 27(6): 1860-1865, 2012.
- Fang B, Zhang M, Tian M, Jiang L, Guo HY and Ren FZ: Bovine lactoferrin binds oleic acid to form an anti-tumor complex similar to HAMLET. *Biochim Biophys Acta* 1841(4): 535-543, 2013.
- Ho CSJ, Rydström A, Trulsson M, Bålfors J, Storm P, Puthia M, Nadeem A and Svanborg C: HAMLET: functional properties and therapeutic potential. *Future Oncol* 8(10): 1301-1313, 2012.



- 5 Nemashkalova EL, Kazakov AS, Khasanova LM, Permyakov EA and Permyakov SE: Structural characterization of more potent alternatives to HAMLET, a tumoricidal complex of  $\alpha$ -lactalbumin and oleic acid. *Biochemistry* 52(36): 6286-6299, 2013.
- 6 Vogel HJ: Lactoferrin, a bird's eye view. *Biochem Cell Biol* 90(3): 233-244, 2012.
- 7 Ruggiero M and Pacini S: The vitamin D axis in chronic kidney disease – State of the art and future perspectives. *European Nephrology* 5(1): 15-19, 2011.
- 8 Yamamoto N, Suyama H, Yamamoto N and Ushijima N: Immunotherapy of metastatic breast cancer patients with vitamin D-binding protein-derived macrophage activating factor (GcMAF). *Int J Cancer* 122(2): 461-467, 2008.
- 9 Yamamoto N, Suyama H, Nakazato H, Yamamoto N and Koga Y: Immunotherapy of metastatic colorectal cancer with vitamin D-binding protein-derived macrophage-activating factor, GcMAF. *Cancer Immunol Immunother* 57(7): 1007-1016, 2008.
- 10 Yamamoto N, Suyama H and Yamamoto N: Immunotherapy for prostate cancer with Gc protein-derived Macrophage-Activating Factor, GcMAF. *Transl Oncol* 1(2): 65-72, 2008.
- 11 Inui T, Kuchiike D, Kubo K, Mette M, Uto Y, Hori H and Sakamoto N: Clinical experience of integrative cancer immunotherapy with GcMAF. *Anticancer Res* 33(7): 2917-2919, 2013.
- 12 Thyer L, Ward E, Smith R, Branca JJ, Morucci G, Gulisano M, Noakes D, Eslinger R and Pacini S: GC protein-derived macrophage-activating factor decreases  $\alpha$ -N-acetylgalactosaminidase levels in advanced cancer patients. *Oncoimmunology* 2(8): e25769, 2013.
- 13 Thyer L, Ward E, Smith R, Branca JJ, Morucci G, Gulisano M, Noakes D and Pacini S: Therapeutic effects of highly purified de-glycosylated gcmf in the immunotherapy of patients with chronic diseases. *Am J Immunol* 9(3): 78-84, 2013.
- 14 Ward E, Smith R, Branca JJ, Noakes D, Morucci G and Thyer L: Clinical experience of cancer immunotherapy integrated with oleic acid complexed with de-glycosylated vitamin d binding protein. *Am J Immunol* 10(1): 23-32, 2014.
- 15 Thyer L, Ward E, Smith R, Fiore MG, Magherini S, Branca JJ, Morucci G, Gulisano M, Ruggiero M and Pacini S: A novel role for a major component of the vitamin D axis: vitamin D binding protein-derived macrophage activating factor induces human breast cancer cell apoptosis through stimulation of macrophages. *Nutrients* 5(7): 2577-2589, 2013.
- 16 Bradstreet JJ, Vogelaar E and Thyer L: Initial observations of elevated alpha-n-acetylgalactosaminidase activity associated with autism and observed reductions from GC protein-macrophage activating factor injections. *Autism Insights* 4: 31-38, 2012.
- 17 Ho VW, Leung K, Hsu A, Luk B, Lai J, Shen SY, Minchinton AI, Waterhouse D, Bally MB, Lin W, Nelson BH, Sly LM and Krystal G: A low carbohydrate, high protein diet slows tumor growth and prevents cancer initiation. *Cancer Res* 71(13): 4484-4493, 2011.
- 18 den Hollander P, Savage MI and Brown PH: Targeted therapy for breast cancer prevention. *Front Oncol* 3: 250, 2013.
- 19 Thun MJ, Jacobs EJ and Patrono C: The role of aspirin in cancer prevention. *Nat Rev Clin Oncol* 9(5): 259-267, 2012.
- 20 Zheng JS, Hu XJ, Zhao YM, Yang J and Li D: Intake of fish and marine n-3 polyunsaturated fatty acids and risk of breast cancer: meta-analysis of data from 21 independent prospective cohort studies. *BMJ* 346: f3706, 2013.
- 21 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
- 22 Smith R, Thyer L, Ward E, Meacci E, Branca JJ, Morucci G, Gulisano M, Ruggiero M, Pacini A, Paternostro F, Di Cesare Mannelli L, Noakes D and Pacini S: Effects of Gc-macrophage activating factor in human neurons; implications for treatment of chronic fatigue syndrome. *Am J Immunol* 9(4): 120-129, 2013.
- 23 Pacini S, Morucci G, Punzi T, Gulisano M and Ruggiero M: Gc protein-derived macrophage-activating factor (GcMAF) stimulates cAMP formation in human mononuclear cells and inhibits angiogenesis in chick embryo chorionallantoic membrane assay. *Cancer Immunol Immunother* 60(4): 479-485, 2011.
- 24 Pacini S, Punzi T, Morucci G, Gulisano M and Ruggiero M: Effects of vitamin D-binding protein-derived macrophage-activating factor on human breast cancer cells. *Anticancer Res* 32(1): 45-52, 2012.
- 25 Knyazeva EL, Grishchenko VM, Fadeev RS, Akatov VS, Permyakov SE and Permyakov EA: Who is Mr. HAMLET? Interaction of human alpha-lactalbumin with monomeric oleic acid. *Biochemistry* 47(49): 13127-13137, 2008.
- 26 Cui CY, Lu WL, Xiao L, Zhang SQ, Huang YB, Li SL, Zhang RJ, Wang GL, Zhang X and Zhang Q: Sublingual delivery of insulin: effects of enhancers on the mucosal lipid fluidity and protein conformation, transport, and *in vivo* hypoglycemic activity. *Biol Pharm Bull* 28(12): 2279-2288, 2005.
- 27 Lu HT, Chen RN, Sheu MT, Chang CC, Chou PY and Ho HO: Rapid-onset sildenafil nasal spray carried by microemulsion systems: *in vitro* evaluation and *in vivo* pharmacokinetic studies in rabbits. *Xenobiotica* 41(7): 567-577, 2011.
- 28 Goto S, Kawata M, Suzuki T, Kim NS and Ito C: Preparation and evaluation of Eudragit gels. I: Eudragit organogels containing drugs as rectal sustained-release preparations. *J Pharm Sci* 80(10): 958-961, 1991.
- 29 Pacini S, Morucci G, Branca JJ, Aterini S, Amato M, Gulisano M and Ruggiero M: Effects of vitamin D<sub>3</sub> and paricalcitol on immature cardiomyocytes: a novel role for vitamin D analogs in the prevention of cardiovascular diseases. *Nutrients* 5(6): 2076-2092, 2013.
- 30 Stuehr DJ and Nathan CF: Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* 169(5): 1543-1555, 1989.
- 31 Nathan CF and Hibbs JB Jr.: Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* 3(1): 65-70, 1991.
- 32 Naska A and Trichopoulou A: Back to the future: the mediterranean diet paradigm. *Nutr Metab Cardiovasc Dis* 24(3): 216-219, 2014.
- 33 Fabris A, Biagioni P, Punzi T, Morucci G, Gulisano M, Pacini S and Ruggiero M: Role of angiotensin-converting enzyme and vitamin d receptor gene polymorphisms in cancer anorexia-cachexia syndrome. *Am J Immunol* 8(3): 65-70, 2012.
- 34 Lucà-Moretti M, Grandi A, Lucà E, Muratori G, Nofroni MG, Mucci MP, Gambetta P, Stimolo R, Drago P, Giudice G and Tamburlin N: Master Amino acid Pattern as substitute for dietary proteins during a weight-loss diet to achieve the body's nitrogen balance equilibrium with essentially no calories. *Adv Ther* 20(5): 282-291, 2003.
- 35 Cicerale S, Lucas LJ and Keast RS: Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. *Curr Opin Biotechnol* 23(2): 129-135, 2012.

- 36 Lakritz JR, Poutahidis T, Levkovich T, Varian BJ, Ibrahim YM, Chatzigiagkos A, Mirabal S, Alm EJ and Erdman SE: Beneficial bacteria stimulate host immune cells to counteract dietary and genetic predisposition to mammary cancer in mice. *Int J Cancer* 135(3): 529-540, 2014.
- 37 Thorat MA and Cuzick J: Role of aspirin in cancer prevention. *Curr Oncol Rep* 15(6): 533-540, 2013.
- 38 Singh S and Gupta AK: Nitric oxide: role in tumour biology and iNOS/NO-based anticancer therapies. *Cancer Chemother Pharmacol* 67(6): 1211-1224, 2011.
- 39 Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo JL, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R and Pittet MJ: Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325(5940): 612-616, 2009.
- 40 Gompf H, Luft FC and Morano I: Nitric oxide synthase upregulation and the predelivery blood pressure decrease in spontaneously hypertensive rats. *J Hypertens* 20(2): 255-261, 2002.
- 41 Hall JE, Granger JP, do Carmo JM, da Silva AA, Dubinion J, George E, Hamza S, Speed J and Hall ME: Hypertension: physiology and pathophysiology. *Compr Physiol* 2(4): 2393-2442, 2012.
- 42 Nonaka K, Onizuka S, Ishibashi H, Uto Y, Hori H, Nakayama T, Matsuura N, Kanematsu T and Fujioka H: Vitamin D binding protein-macrophage activating factor inhibits HCC in SCID mice. *J Surg Res* 172(1): 116-122, 2012.
- 43 Partridge SC, Gibbs JE, Lu Y, Esserman LJ, Tripathy D, Wolverton DS, Rugo HS, Hwang ES, Ewing CA and Hylton NM: MRI measurements of breast tumor volume predict response to neoadjuvant chemotherapy and recurrence-free survival. *Am J Roentgenol* 184(6): 1774-1781, 2005.
- 44 Fontana A, Spolaore B and Polverino de Laureto P: The biological activities of protein/oleic acid complexes reside in the fatty acid. *Biochim Biophys Acta* 1834(6): 1125-1143, 2013.
- 45 Gregory KJ, Zhao B, Bielenberg DR, Dridi S, Wu J, Jiang W, Huang B, Pirie-Shepherd S and Fannon M: Vitamin D binding protein-macrophage activating factor directly inhibits proliferation, migration, and uPAR expression of prostate cancer cells. *PLoS One* 5(10): e13428, 2010.
- 46 Pacini S, Morucci G, Punzi T, Gulisano M, Ruggiero M, Amato M and Aterini S: Effect of paricalcitol and GcMAF on angiogenesis and human peripheral blood mononuclear cell proliferation and signaling. *J Nephrol* 25(4): 577-581, 2012.
- 47 Rockett KA, Brookes R, Udalova I, Vidal V, Hill AV and Kwiatkowski D: 1,25-Dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of Mycobacterium tuberculosis in a human macrophage-like cell line. *Infect Immun* 66(11): 5314-5321, 1998.
- 48 Choudhari SK, Chaudhary M, Bagde S, Gadbail AR and Joshi V: Nitric oxide and cancer: a review. *World J Surg Oncol* 11: 118, 2013.
- 49 Borutaite V and Brown G: What else has to happen for nitric oxide to induce cell death? *Biochem Soc Trans* 33(Pt 6): 1394-1396, 2005.
- 50 Storm P, Aits S, Puthia MK, Urbano A, Northen T, Powers S, Bowen B, Chao Y, Reindl W, Lee DY, Sullivan NL, Zhang J, Trulsson M, Yang H, Watson JD and Svanborg C: Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis. *Oncogene* 30(48): 4765-4779, 2011.

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