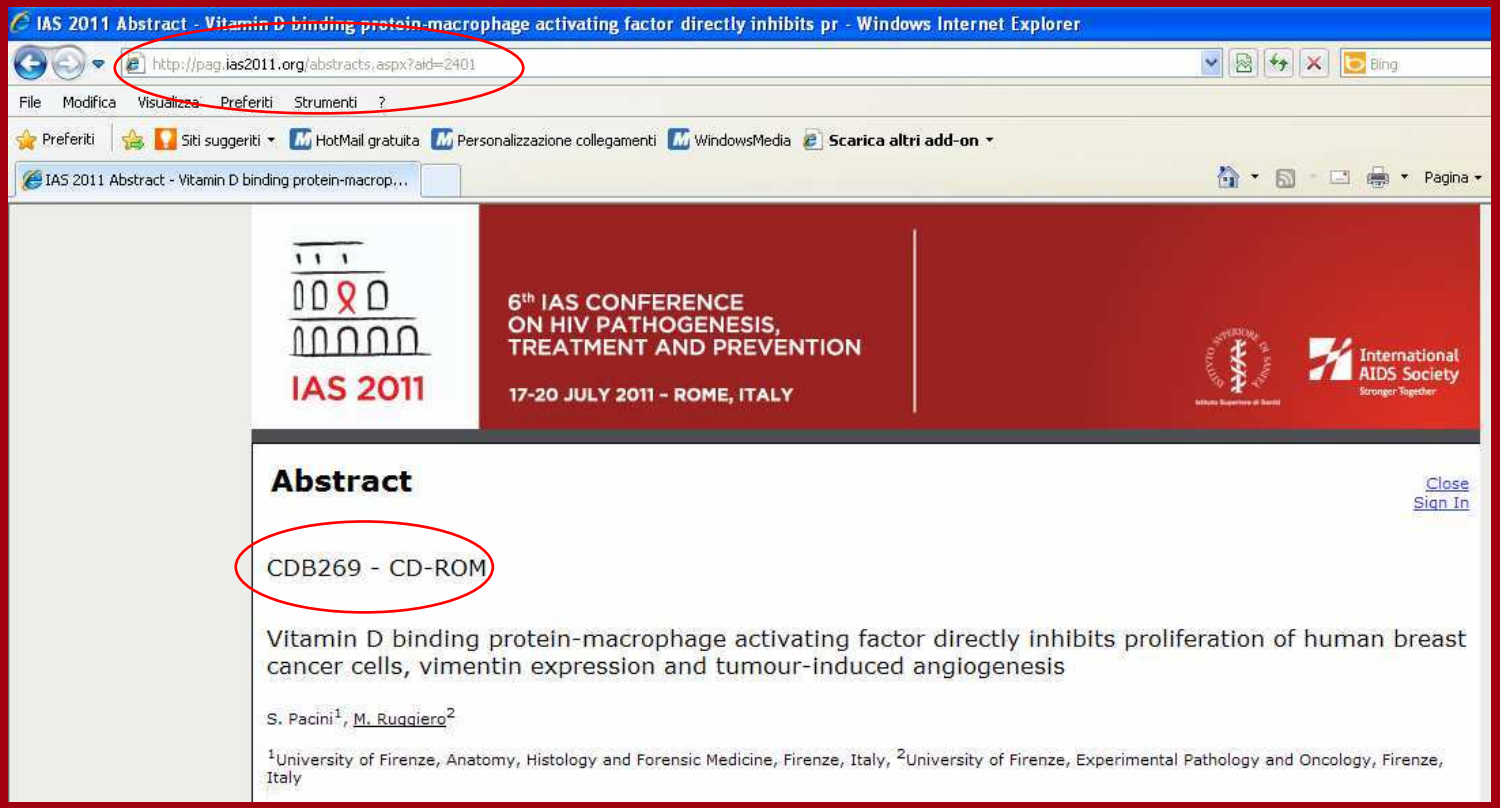
The following presentation

- Has been selected as an e-poster at the Sixth IAS Conference on HIV pathogenesis, treatment and prevention, Rome, July 17-20, 2011.
- It is published in the CD-ROM distributed to all participants and it is available at http://pag.ias2011.org/EPosterHandler.axd?aid=2401



Abstract

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Vitamin D binding protein-macrophage activating factor directly inhibits proliferation of human breast cancer cells, vimentin expression and tumour-induced angiogenesis

S. Pacini[1], M. Ruggiero[2]

[1]University of Firenze, Anatomy, Histology and Forensic Medicine, Firenze, Italy, [2]University of Firenze, Experimental Pathology and Oncology, Firenze, Italy

Background: The incidence of HIV infection is rising in women and even though its impact on breast cancer incidence is still under investigation, it is well assessed that patients with HIV infection present with more advanced stage and aggressive disease, and they also have poor chemotherapy tolerance. Vitamin D binding protein-macrophage activating factor (DBP-MAF) has been successfully used in immunotherapy of HIV-infected patients (J Med Virol 81:16-26, 2009). Since HIV infection and breast cancer can coexist in women, in this study we evaluated the effects of DBP-MAF on human breast cancer cell proliferation and tumour-induced angiogenesis.
Methods: DBP-MAF was obtained from www.gcmaf.eu. Assessment of MCF-7 (human breast cancer) cell proliferation was determined by Calbiochem Rapid Cell Proliferation Kit. MCF-7 cells were also studied by scanning and conventional microscopy. MCF-7-induced angiogenesis was studied in chick embryo chorionallantoic membrane (CAM) assay.
Results: DBP-MAF (0.4-40 ng/ml, incubated for 72 h) inhibited MCF-7 cell proliferation in a dose-dependent manner. Vitamin D also inhibited MCF-7 cell proliferation and the effects of vitamin D and DBP-MAF were additive. DBP-MAF-treated cells were significantly smaller and inhomogeneous as if processes of shrinkage had occurred. Cytoplasm and nucleus appeared irregular as if fragmented. Cellular debris could be observed as well as apoptotic bodies. Vimentin expression was reduced following DBP-MAF treatment. It is worth noting that increased vimentin expression is considered a hallmark of progression of breast cancer due to tumour cells losing their epithelial features and gaining mesenchymal properties. DBP-MAF inhibited MCF-7-induced neo-angiogenesis in CAM assay, another critical step in breast cancer progression.
Conclusion: These results demonstrate that DBP-MAF, in addition to stimulating macrophages, directly inhibits human breast cancer cell growth in vitro. Therefore, administration of DBP-MAF to HIV-infected women could provide the dual benefit of immunotherapy of HIV infection and prevention of breast cancer progression.
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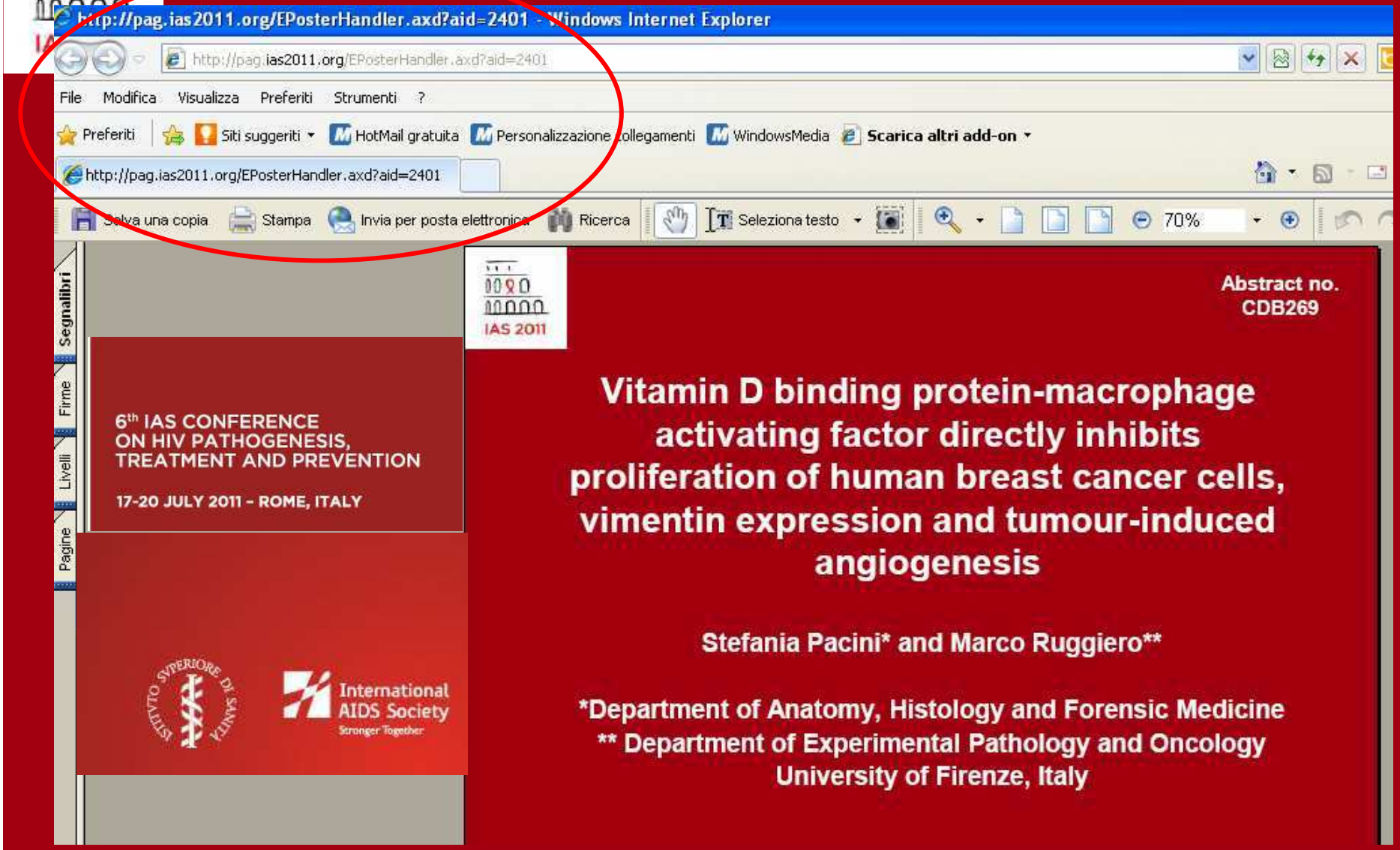
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Abstract no.
CDB269
Vitamin D binding protein-macrophage activating factor directly inhibits proliferation of human breast cancer cells, vimentin expression and tumour-induced angiogenesis
Stefania Pacini* and Marco Ruggiero**
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IAS 2011

Abstract no.
CDB269

Vitamin D binding protein-macrophage activating factor directly inhibits proliferation of human breast cancer cells, vimentin expression and tumour-induced angiogenesis

Stefania Pacini* and Marco Ruggiero**

*Department of Anatomy, Histology and Forensic Medicine
** Department of Experimental Pathology and Oncology
University of Firenze, Italy

Background (1)

- Breast cancer is of particular importance among non-AIDS defining cancers. Although breast cancer risk is significantly lower for women with HIV infection compared to the general population (PLoS One 16:e14349, 2010), patients with HIV infection present with more advanced stage and aggressive breast cancer, and they also have poor chemotherapy tolerance.
- This led us to the search for an alternative approach targeting both immunodeficiency and cancer, and we focussed on vitamin D binding protein-macrophage activating factor (DBP-MAF, also known as GcMAF), a factor that has been successfully used in immunotherapy of HIV-infected (J Med Virol 81:16-26, 2009), and breast cancer patients (Int J Cancer 122:461-7, 2008).

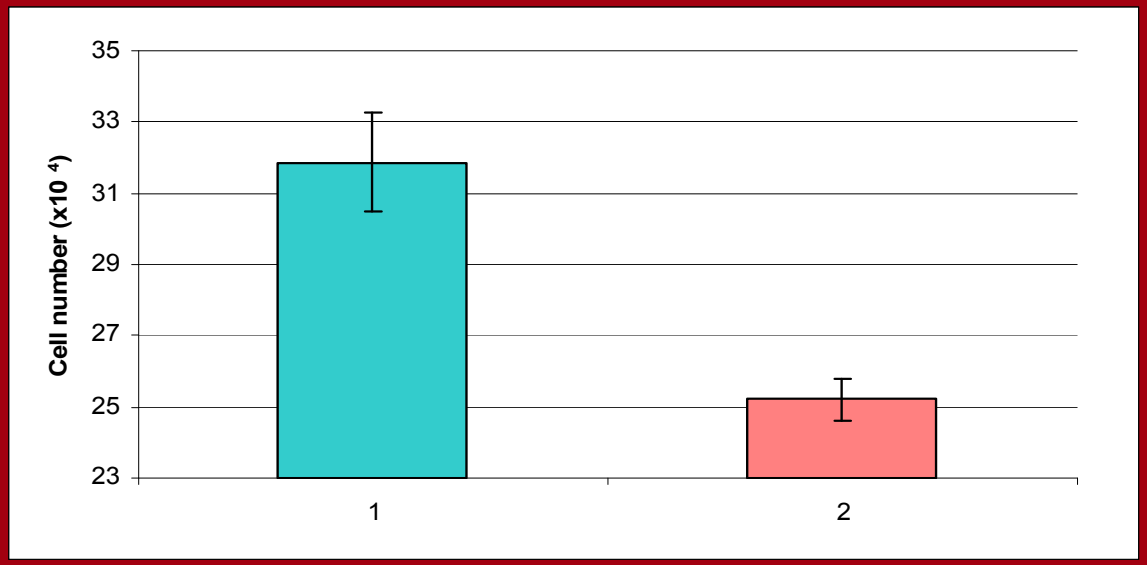
Background (2)

- Here we demonstrate that, in addition to the known immune-stimulatory effects, DBP-MAF also directly inhibits human breast cancer cell proliferation, reverses their malignant phenotype, and inhibits cancer cell-stimulated angiogenesis.
- Here we also report the effects of DBP-MAF on the immune system of HIV/AIDS patients.
- Finally, we describe the effects of an original probiotic preparation, putatively containing DBP-MAF, on the immune system.

Methods

- Gc-protein (*i.e.* the precursor of DBP-MAF), and DBP-MAF were donated by www.gcmaf.eu.
- MCF-7 (human breast adenocarcinoma) cells were from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Italy.
- Cell proliferation was determined by cell count.
- Angiogenesis was studied by chick embryo chorioallantoic membrane (CAM) assay.
- Cell morphology was studied by light microscopy.
- Vimentin expression was studied by immunohistochemistry and western blot analysis.
- In order to avoid artifacts due to non-specific protein interactions, the effects of DBP-MAF on cultured MCF-7 cells were compared to those of Gc-protein administered at the same concentration.

Results (1)



DBP-MAF-induced inhibition of MCF-7 cell proliferation

Cells were seeded at semi-confluence in medium containing 1% FCS and 0.4 ng/ml Gc-protein (column 1), or DBP-MAF (column 2). Cells were counted after 72 h. Results are expressed as means±SEM (n=4).

Maximal inhibitory effects on cell proliferation were observed with 0.4 ng/ml DBP-MAF concentration. This concentration was similar to that required to stimulate human peripheral blood mononuclear cells (Cancer Immunol Immunother 60:479-85, 2011).

Results (2)

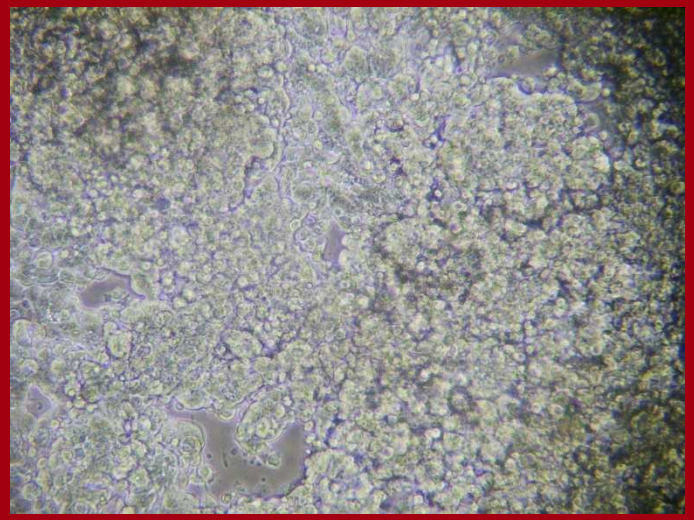
Quantitative evaluation of angiogenesis on CAM assay.
Effects of DBP-MAF on MCF-7-stimulated angiogenesis

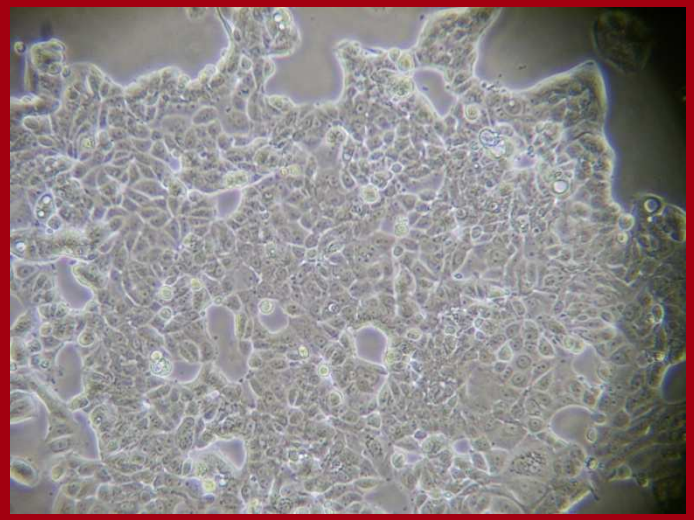
Experimental point	Circumfocal microvessel number
PBS	15.3 ± 1.4
DBP-MAF	16.4 ± 1.8
MCF-7	28.5 ± 1.3
MCF-7 + DBP-MAF	16.7 ± 1.0*

The average number of blood vessels derived from scoring small (< 1 mm dia.), large (> 1 mm dia.), and tortuous microvessels. DBP-MAF; 40 ng/ml. Data are reported as means ± SEM (n=18). * $p < 0.02$ *vs* MCF-7.

It is worth noting that DBP-MAF concentration required to achieve full inhibition of cancer cell-stimulated angiogenesis was higher than that required to inhibit MCF-7 cell proliferation or to stimulate human peripheral blood mononuclear cells (Cancer Immunol Immunother 60:479-85, 2011).

Results (3)





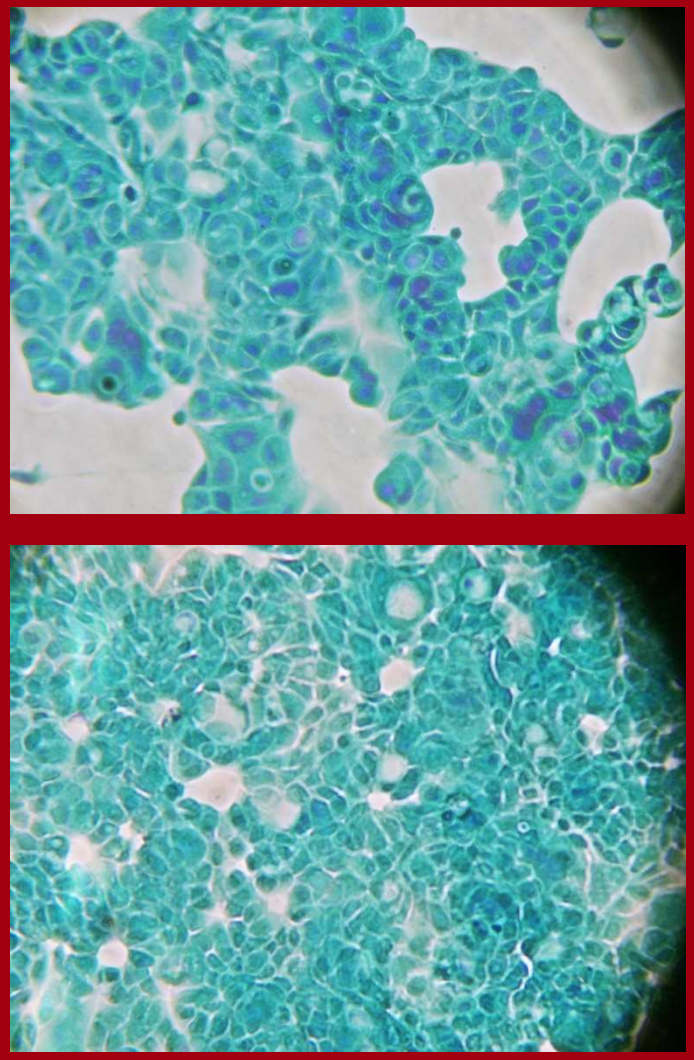
- Phase contrast light microscopy of MCF-7 living cells. Cells did not undergo any treatment, *i.e.* washing, fixation or staining. Magnification 300x.

  Upper panel; cells treated with 40 ng/ml Gc-protein for 72 h. Cells did not show contact inhibition and formed tumour clusters.

  Lower panel; cells treated with 40 ng/ml DBP-MAF for 72 h. Cells grew in monolayer and no clusters could be observed. Cells were regularly polygonal and uniform in morphology and size.

Results (4)

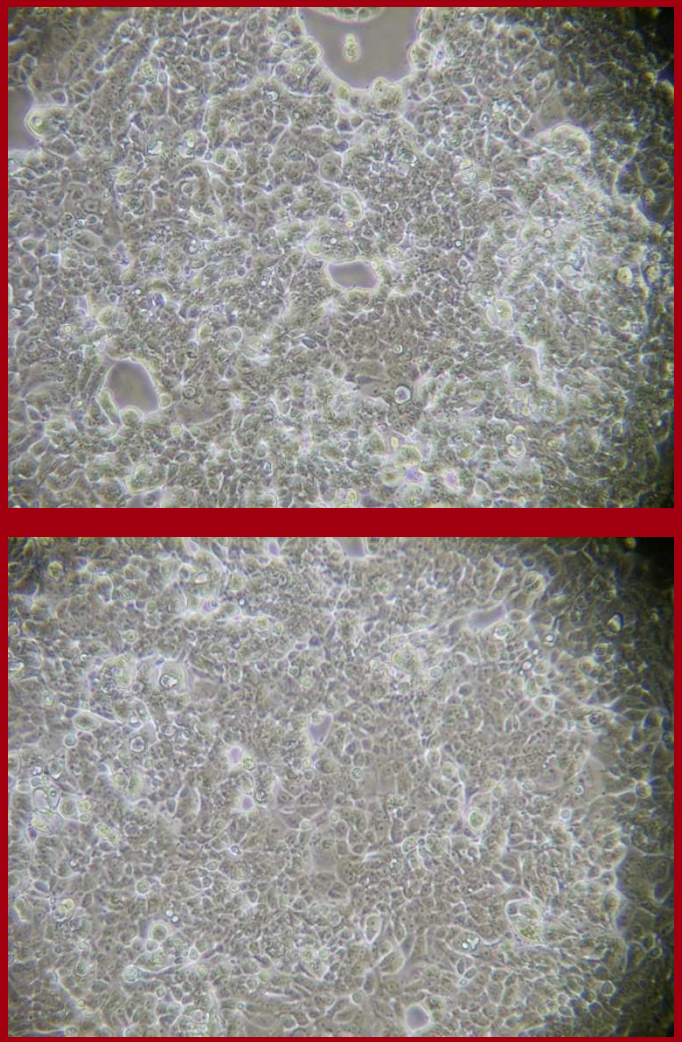
Papanicolaou stain. Magnification 600x.



- Upper panel; MCF-7 cells treated with 40 ng/ml Gc-protein for 72 h. Cells grew one on top of the other forming typical tumour clusters. Cell size, morphology and staining were inhomogeneous. Large empty spaces between clusters indicate poor adherence to the well surface.

- Lower panel; cells treated with 40 ng/ml DBP-MAF for 72 h. Cells grew in monolayer and were smaller, regularly polygonal and uniform in size and morphology. Cells appeared to be well adherent to each other and to the well surface.

Results (5)



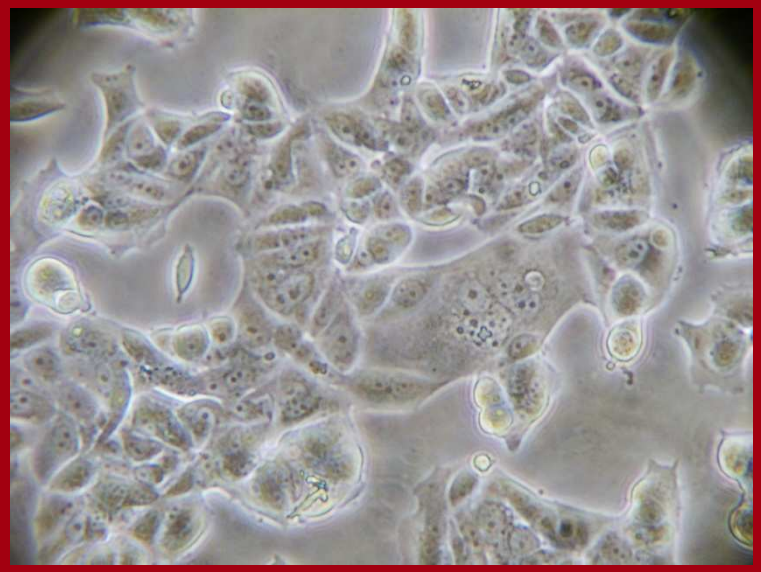
- DBP-MAF concentration required to induce major morphological changes was higher than that required to inhibit cell proliferation and identical to that required to inhibit angiogenesis.
- It is worth noting, however, that minor morphological changes could be observed also at lower concentration.

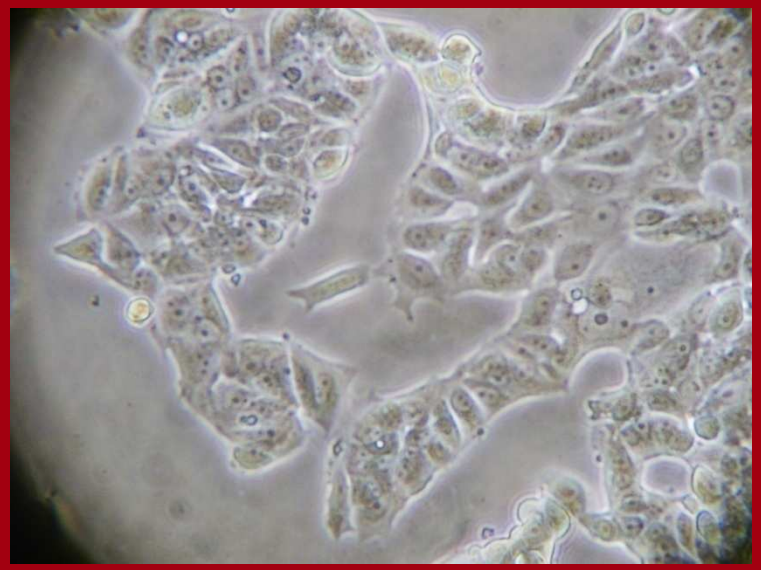
Phase contrast microscopy of MCF-7 cells, 300x.

Upper panel; MCF-7 cells treated with 0.4 ng/ml Gc-protein for 72 h.

Lower panel; cells treated with 0.4 ng/ml DBP-MAF for 72 h.

Results (6)



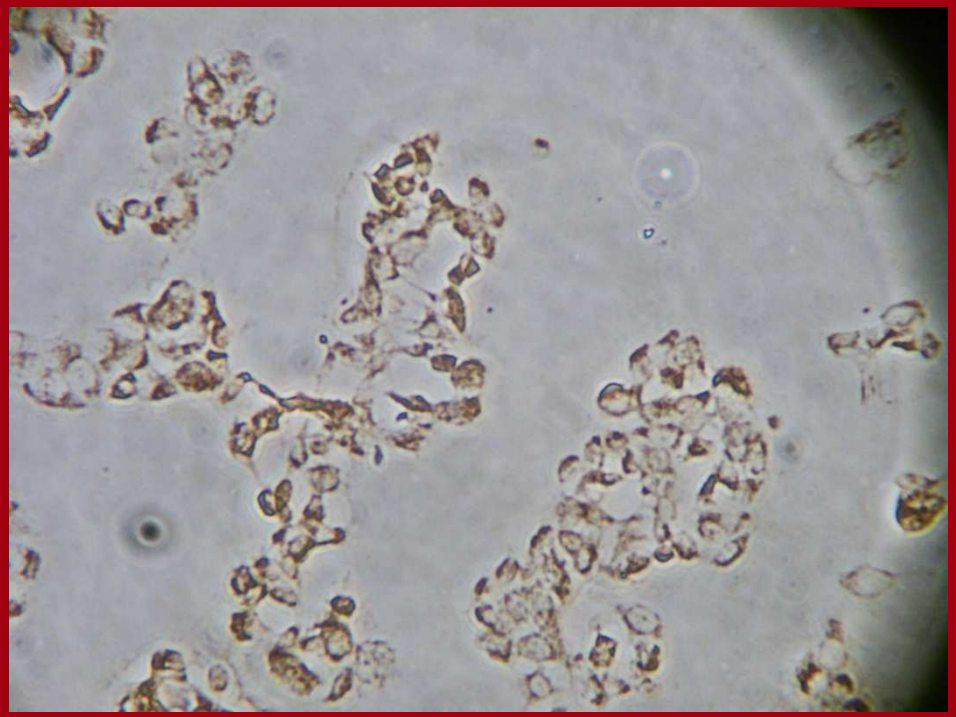


- Morphological changes could also be observed after 24 h treatment.
- Phase contrast microscopy of MCF-7 cells, 600x.

  Upper panel; MCF-7 cells treated with 40 ng/ml Gc-protein for 24 h.

  Lower panel; cells treated with 40 ng/ml DBP-MAF for 24 h.

Results (7)

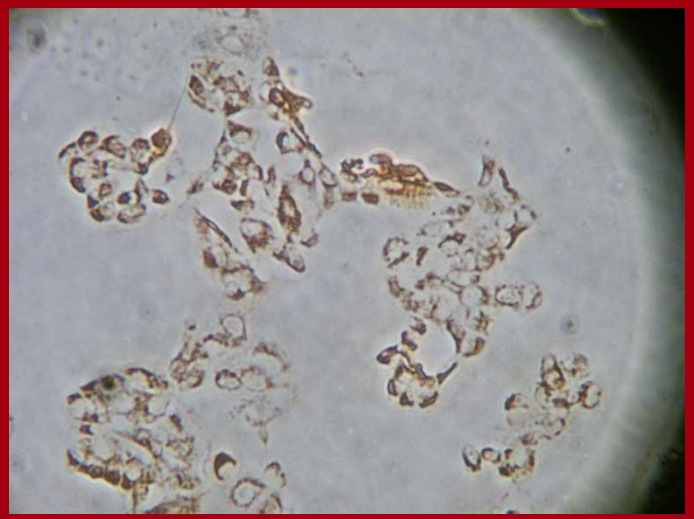


Strong vimentin expression (brown) in MCF-7 cells treated with 40 ng/ml Gc-protein for 72 h. Immunohistochemical analysis, 600x.

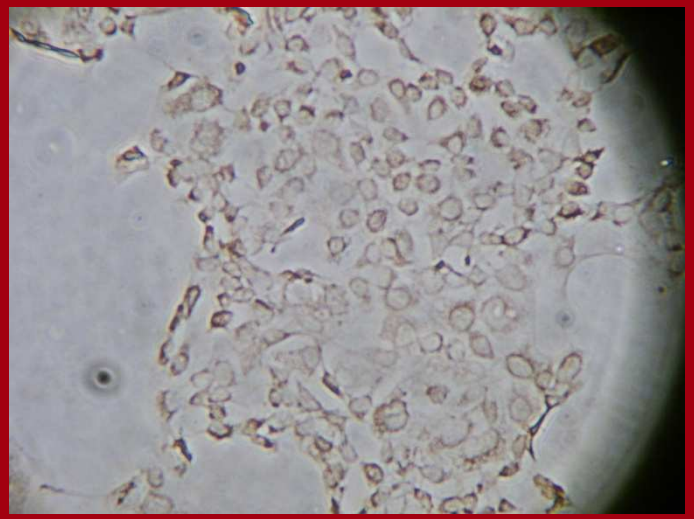
- DBP-MAF-induced morphological changes can be interpreted as if DBP-MAF reverted cancer cell malignant phenotype, a phenomenon confirmed by the study of vimentin expression.
- Vimentin expression is considered a hallmark of human breast cancer progression. In fact, during progression toward a more malignant phenotype, the cell intermediate filament status changes from a keratin-rich to a vimentin-rich network in a process termed “epithelial-mesenchymal transition” (Cells Tissues Organs 185:191-203, 2007).

Results (8)

Gc-protein



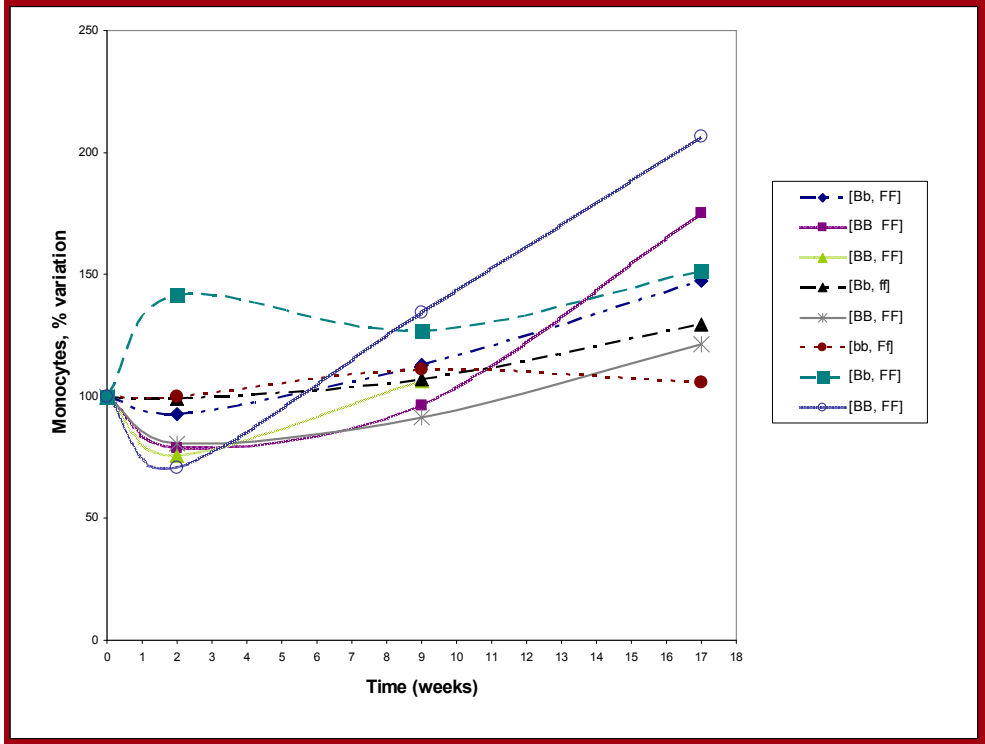
DBP-MAF



- Immunohistochemical analysis, demonstrated that exposure to 40 ng/ml DBP-MAF for 72 h significantly decreased vimentin expression in MCF-7 cells.
- These data, confirmed by western blot analysis (not shown), are consistent with DBP-MAF-induced reversal of epithelial-mesenchymal transition.

Results (9)

Effects of DBP-MAF on the immune system *in vivo*



Peripheral blood monocyte count before treatment (Time 0) was considered 100%. Last determination was performed two weeks after the last injection. Each symbol refers to a patient whose VDR genotype is reported on the right. The last results of one patient were not available.

- Eight HIV/AIDS patients were treated with 100 ng/week DBP-MAF (www.gcmaf.eu) i.v. for 15 weeks.
- During treatment, patients did not assume antiretroviral drugs.
- Blood monocyte count rose in six patients.
- These results are consistent with the effects of DBP-MAF described in Immunol Cell Biol 76:237-44, 1998.
- Individual response appeared to be associated with vitamin D receptor (VDR) gene polymorphisms (*Bsm*I and *Fok*I).

Preliminary case reports courtesy of Dr. Santos-Koenig, Vienna, Austria.

Results (10)

Effects of a probiotic preparation putatively containing DBP-MAF on the immune system *in vivo*

We tested an original milk-derivative containing microorganisms introduced in order to maximize natural DBP-MAF production. We hypothesized that this natural DBP-MAF, once ingested, activated the Mucosa-Associated Lymphoid Tissue (MALT) widely diffused in the walls of the entire gastrointestinal tract.

- Enzymes of certain strains of microorganisms contained in yogurt and kefir are able to convert milk Gc-protein into active DBP-MAF.
- It is known that kefir modulates the immune response in mice, increasing the phagocytic activity (*i.e.* activating) of peritoneal and pulmonary macrophages (Immunobiology 211:149-56, 2006).
- It is also known that probiotic yogurt consumption is associated with an increase of CD4 count among people living with HIV/AIDS (J Clin Gastroenterol 44:e201-5, 2010).

Results (11)

- Members of the research team consumed 125 ml/day of the original probiotic preparation for three weeks.
- Participants did not assume any drug or supplement and did not modify their usual diet and lifestyle.
- Blood analyses were performed two weeks before beginning consumption, and after three week consumption.
- After three week consumption, CD4 count dramatically increased in those of us who started with low CD4 count (subject # 1), or abnormal CD4/CD8 ratio (subject # 2).
- These effects appeared to be associated with VDR gene polymorphisms.

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1, before consumption.

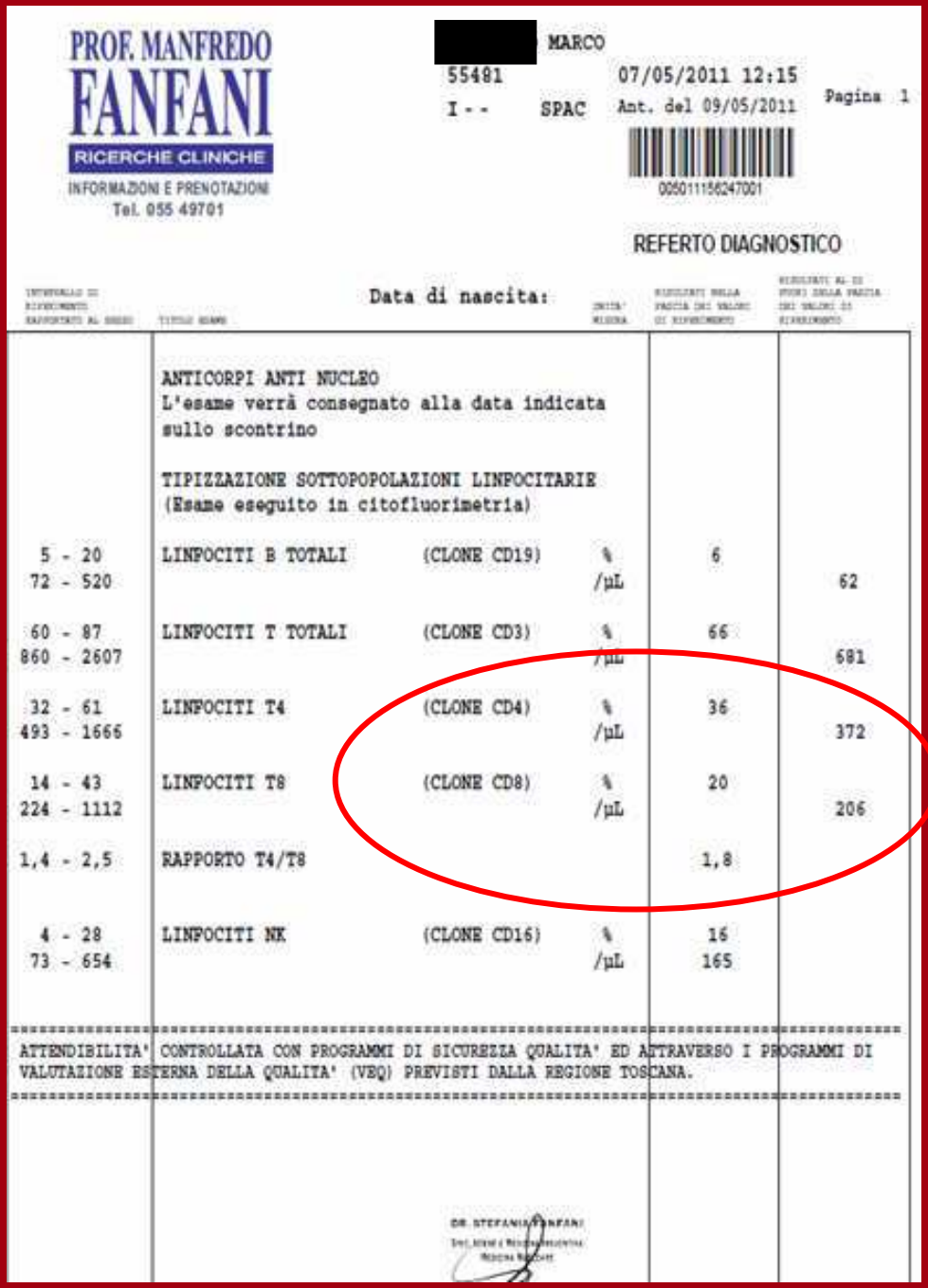
CD4: 372

CD8: 206

CD4/CD8: 1.8

VDR genotype: bb, FF

Reference values
CD4: 493-1666
CD8: 224-1112
CD4/CD8: 1.4-2.5



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4 - 28 73 - 654	LINFOCITI NK	(CLONE CD16)	% /µL	16 165	

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DR. STEFANO FANFANI

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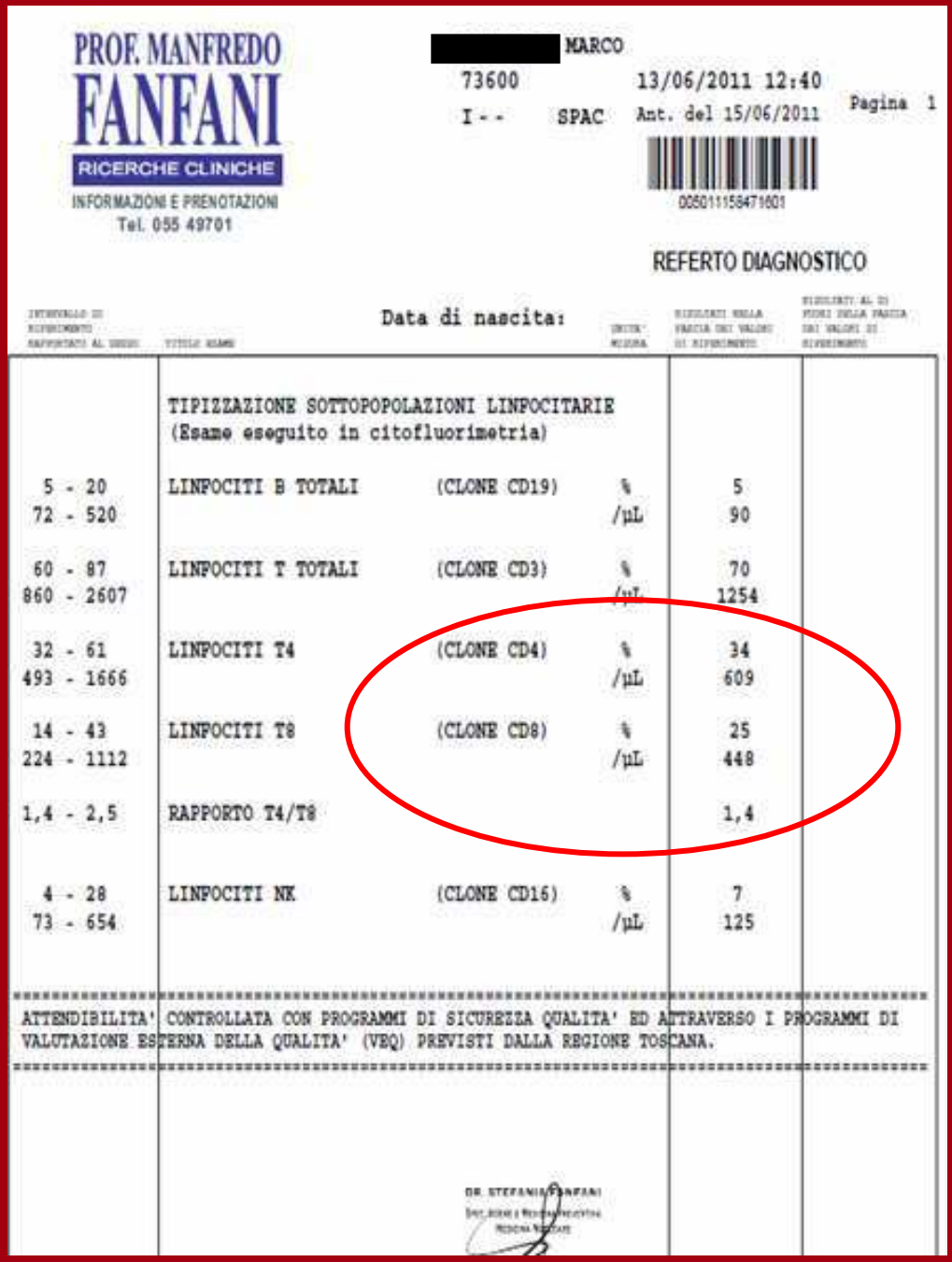
1, after consumption.

CD4: 609

CD8: 448

CD4/CD8: 1.4



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DR. STEFANIA FANFANI

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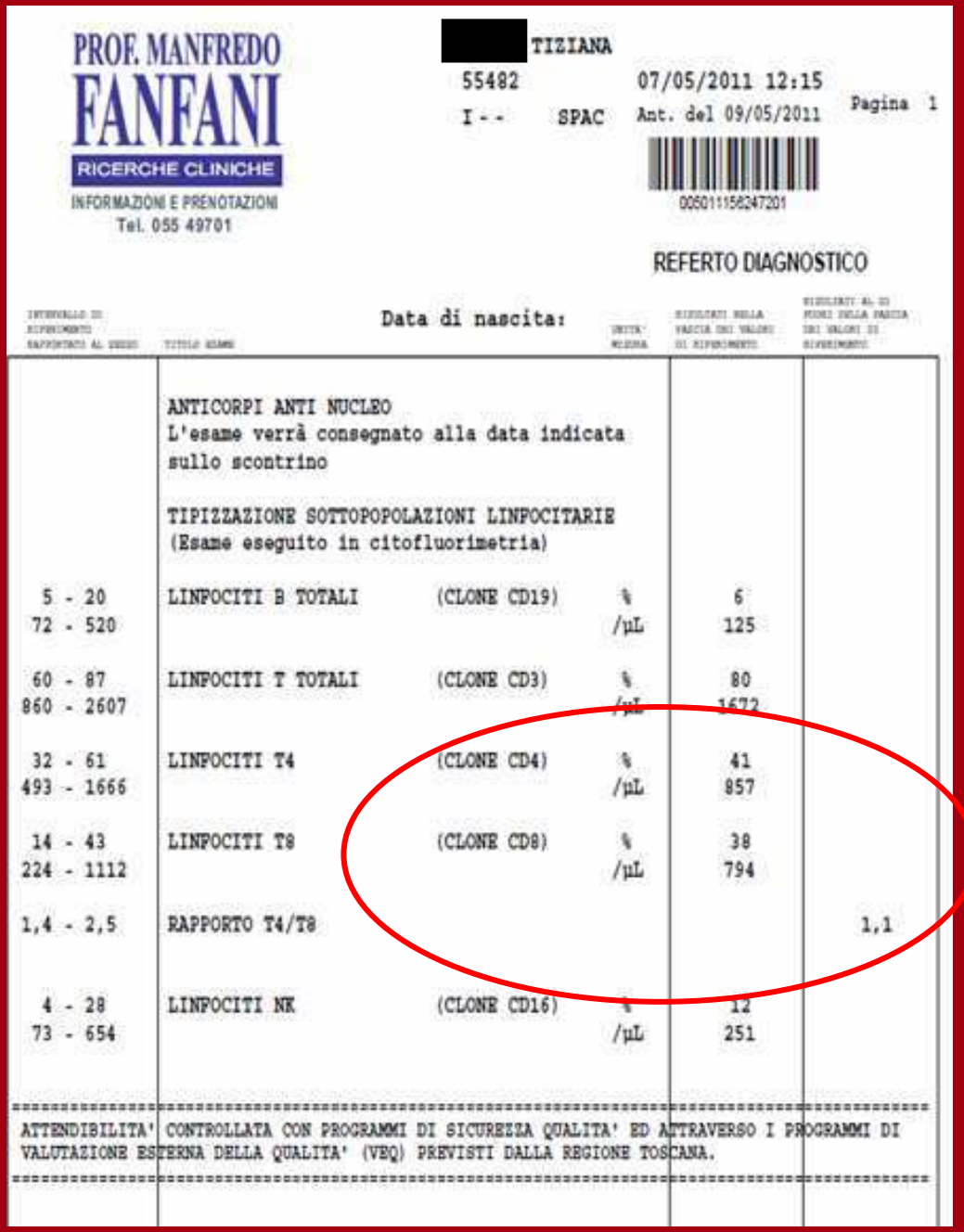
2, before consumption.

CD4: 857

CD8: 794

CD4/CD8: 1.1

VDR genotype: Bb, FF



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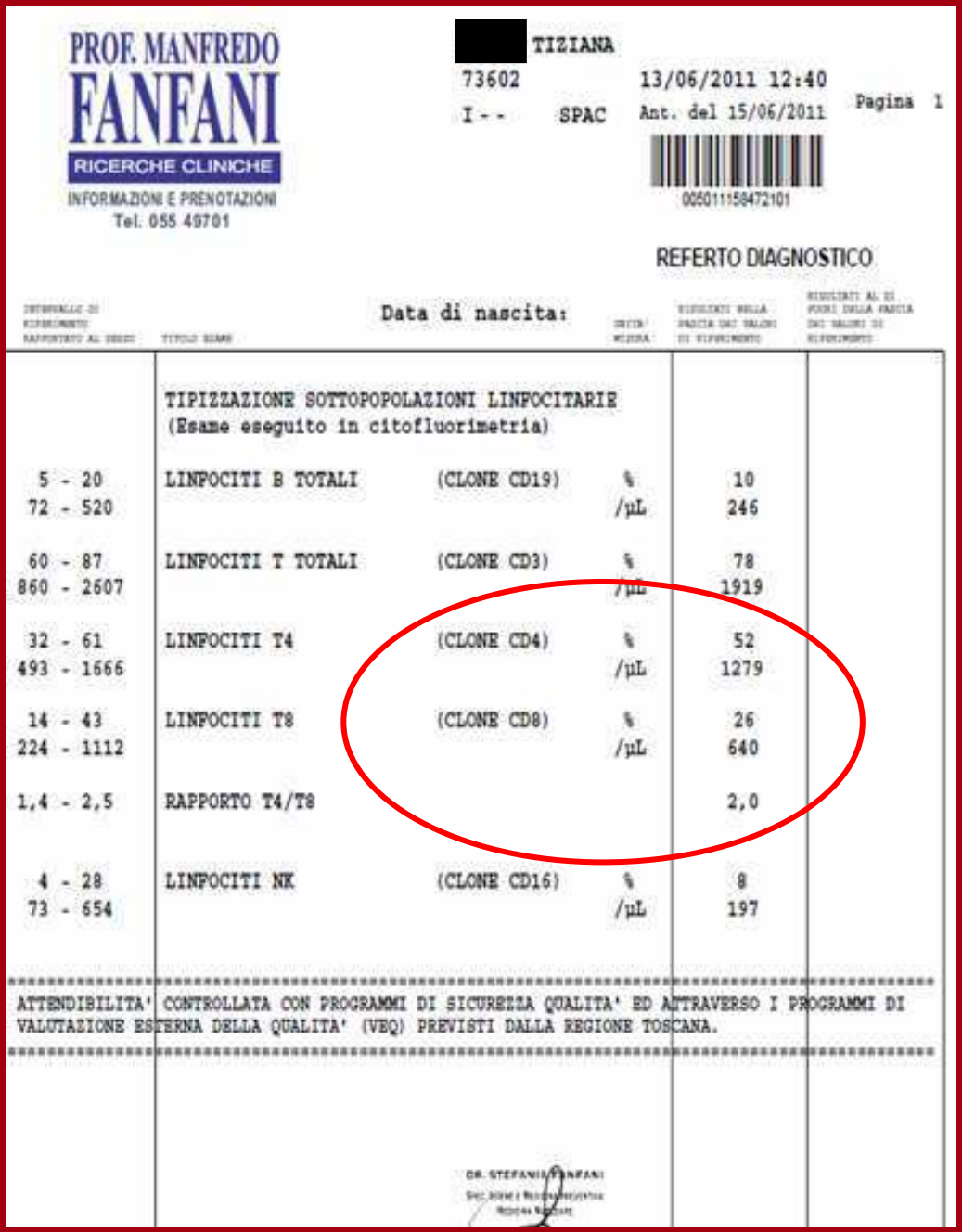
2, after consumption.

CD4: 1279

CD8: 640

CD4/CD8: 2.0



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1,4 - 2,5	RAPPORTO T4/T8			2,0
4 - 28 73 - 654	LINFOCITI NK	(CLONE CD16)	% /µL	8 197

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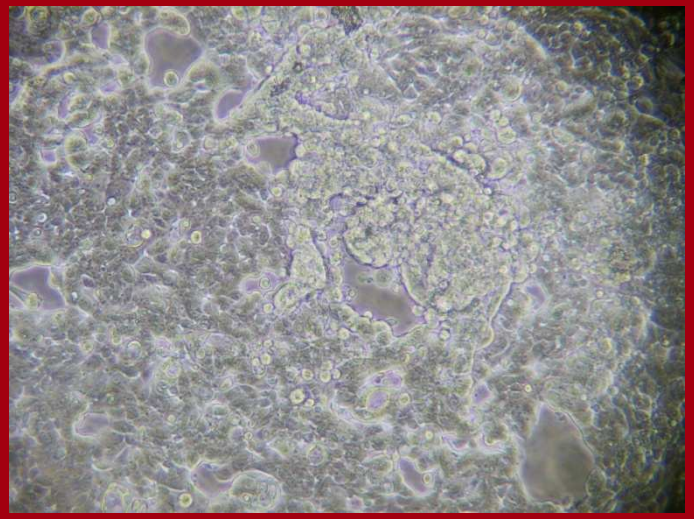
Please notice, this slide has beed added <u>after</u> the conference and it <u>is not</u> published in the CD-ROM

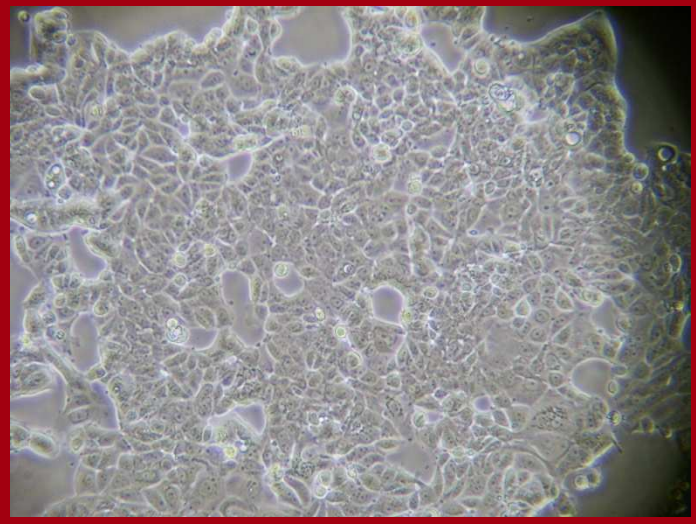
- "To put these increases in perspective, studies have estimated that **<u>ART increases the average annual CD4 count by 90 cells</u>**/ µl versus an average decline of 20–50 cells/µl/year without treatment."

- Gregor Reid, Lawson Health Research Institute; Departments of Microbiology & Immunology and Surgery; The University of Western Ontario; London, Ontario Canada. The potential role for probiotic yogurt for people living with hiv/aids. Gut Microbes 1:6, 411-414; November/December 2010; © 2010 Landes Bioscience.

Conclusion



Gc-protein 40 ng/ml, 72 h



DBP-MAF 40 ng/ml, 72 h

- Our data demonstrate that DBP-MAF exerts multiple effects on human breast cancer cells; these anti-cancer effects, coupled with the known immune-stimulatory effects, could prove useful in treatment of non-AIDS defining cancers in HIV-positive patients.
- Future directions of our research involve further development of the original probiotic preparation, putatively containing DBP-MAF, that shows promising immune-stimulatory effects.