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Spa therapy induces clinical improvement and protein changes in patients with chronic back pain

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SUMMARY

This study is primarily aimed at assessing serum changes on a large panel of proteins in patients with chronic back pain following spa therapy, as well as evaluating different spa therapy regimens as a preliminary exploratory clinical study.

Sixty-six patients with chronic back pain secondary to osteoarthritis were randomly enrolled and treated with daily mud packs and bicarbonate-alkaline mineral water baths, or a thermal hydrotherapy rehabilitation scheme, the combination of the two regimens or usual medication only (control group), for two weeks. Clinical variables were evaluated at baseline, after 2 and 12 weeks. One thousand serum proteins were tested before and after a two-week mud bath therapy.

All spa treatment groups showed clinical benefit as determined by improvements in VAS pain, Roland Morris disability questionnaire and neck disability index at both time points. The following serum proteins were found greatly increased (≥2.5 fold) after spa treatment: inhibin beta A subunit (INHBA), activin A receptor type 2B (ACVR2B), angiopoietin-1 (ANGPT1), beta-2-microglobulin (B2M), growth differentiation factor 10 (GDF10), C-X-C motif chemokine ligand 5 (CXCL5), fibroblast growth factor 2 (FGF2), fibroblast growth factor 12 (FGF12), oxidized low density lipoprotein receptor 1 (OLR1), matrix metallopeptidase 13 (MMP13). Three proteins were found greatly decreased (≤0.65 fold): apolipoprotein C-III (Apoc3), interleukin 23 alpha subunit p19 (IL23A) and syndecan-1 (SDC1).

Spa therapy was confirmed as beneficial for chronic back pain and proved to induce changes in proteins involved in functions such as gene expression modulation, differentiation, angiogenesis, tissue repair, acute and chronic inflammatory response.

Key words: *Back pain; osteoarthritis; balneology; mud therapy; rehabilitation.*

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INTRODUCTION

Salus per aquam (spa) therapy represents a popular treatment for several rheumatic diseases, such as axial and peripheral osteoarthritis, fibromyalgia and extra-articular rheumatisms (1-6), but also for chronic arthritis as adjunctive therapy, such as in spondyloarthritis and rheumatoid arthritis (7-10).

The therapeutic beneficial effects of spa treatments are believed to be mainly due to the intrinsic water physical/chemical features. Thermal mineral water contains solutes such as cations (*i.e.* calcium, magnesium, sodium and potassium) and anions (bicarbonates, sulphates, chloride) at a minimum concentration of at least 1 g/L (11). Thermal water effects appear therefore mediated by the physical and chemical properties of water by means of mechanical and thermal effects together with the absorption of mineral solutes which are believed to contribute to the beneficial effects on the inflammatory pathway and bone metabolism, recently summarized by Fioravanti et al. (12) and Cozzi et al. (13). A very popular way to deliver balneological therapy is also by means of mud pack, which is defined as a natural mixture of

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mineral water with organic or inorganic material (*i.e.* clay) and delivered in the form of mud wrap (14).

Spinal diseases and chronic back pain (15) are the most prevalent musculoskeletal conditions which affect, during lifetime, the vast majority of the population. Back pain is regarded as chronic when it lasts longer than two/three months (16). The scientific literature dedicated to spa therapy in chronic back pain has been reviewed in the past (17, 18). The beneficial effects have also been underlined by Balogh et al. (19) and by Kulisch et al. (20). Tefner et al. (21) have conducted a single blind randomized controlled trial demonstrating the beneficial effects of balneotherapy compared to tap water, while more recently Gáti et al. (22) have confirmed the therapeutic effects of the calcium-magnesium-bicarbonate thermal water, both studies in chronic low back pain.

A less frequent cause of chronic back pain is represented by ankylosing spondylitis (AS) (23), a systemic inflammatory chronic disease affecting primarily the spine and affecting a younger population. In the later stage of disease, AS patients often present new bone formation such as bridging syndesmophytes which may result in a stiff spine and the development of a clinical picture characterized by *mechanical back pain* rather than the previous *inflammatory back pain*.

Given the solid background present in the literature, the primary aim of this study was to evaluate serum changes on a large spectrum (one thousand) of proteins in patients with chronic back pain following MBT therapy. Furthermore, we also performed a preliminary exploratory study testing the clinical effects of different spa therapy regimens.

MATERIALS AND METHODS

A cohort of 66 chronic back pain secondary to axial osteoarthritis (axOA) patients were included in this study. 46 patients (31F/15M) underwent spa therapy and 20 patients (13F/7M), as a control group, were treated according to their usual regime. Furthermore, 11 patients with chronic back pain secondary to axial spondyloarthritis (ankylosing spondylitis), with inactive inflammatory disease, were also treated as parallel group. The study was conducted at the *Antiche Terme di Sardara* (VS Sardegna - Italy), which provided all the facilities for mud treatment and balneotherapy (MBT) and the thermal hydrotherapy rehabilitation scheme (HRS). Ethical approval was granted by the local NHS authority ASL6 prot. 2012/0021248, and was in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients participated in the study on a voluntary basis, providing informed consent, according to good clinical practice. The mean age was 64.5±11.4 and BMI was 24.2±2.0 in the axOA spa therapy group; the mean age was 63.3±6.9 and the BMI 22.4±2.6 in the axOA control group. In the AS group the sex ratio (2F/9M) and mean age 50.5±10.8 were lower in accordance with the epidemiology of the disease and the BMI was 23.9±2.5. All patients were on stable medication. Patients with the following co-pathologies were not included in the study: evolving cardiovascular diseases, cancer, infection, uncontrolled diabetes mellitus, severe lung or kidney disease, joint swelling or effusion, arthroprosthesis carrier, neurologic severe diseases, peripheral vascular diseases, any medical condition or pharmacologic treatment that, according to the enrolling physician, should contraindicate participation in the study. Furthermore, subjects who had spa treatments in the previous 6 months were also excluded. All patients were Caucasian and were resident in the nearby area of the spa resort.

The patients were treated daily for two weeks according to two different thermal therapeutic interventions:

1) a combination of mud packs applied on the body surface at girdles and spine for 20 min at an initial temperature of 45°C followed by bicarbonate-alkaline mineral water bath (Table I) at 38°C for 10 min, and by 45 min of rest (mud bath therapy scheme, MBT), for a total of 12 applications carried out over a period of 2 weeks; and/or

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2) a thermal hydrotherapy rehabilitation scheme (HRS) which included stretches and strengthening exercises, floating or standing in the thermal pool (water temperature 34°C). Upper and lower limbs, girdles, lower back, trunk and neck were involved in the exercise program. 45- to 60-minute sessions according to personal physical endurance were supervised by professional rehabilitation staff daily, 12 times over a two-week period.

Group A (12 patients with axOA) was treated with MBT only, group B (16 patients with axOA) was treated by the combination of MBT and HRS, group C (18 patients with axOA) was treated with HRS only. Twenty axOA patients had no thermal treatments, remained in their usual medications and were included in the control group. Patients were recruited randomly according to Zelen (24), following referral from a general practitioner (GP). The distinct group of 11 AS patients was treated by HRS only.

Clinical assessment at baseline and follow-up visits at two weeks (T2W) and 12 weeks (T12W) included detailed objective examination and medical history, as well as evaluation of specific clinical domains by means of the following instruments: visual analogue scale (VAS) for pain (0=no pain, 10=maximum pain), short form health survey of 36 items (SF-36) (25) referring to physical and mental components (quality of life) in all patients; Roland and Morris Disability Questionnaires (26) and Neck Disability Index (27), for axial involvement in axOA chronic back pain. Bath Ankylosing Spondylitis Metrology/Functional/Disease activity indexes (BASMI, BASFI and BASDAI) were administered to AS patients (28).

In order to study protein changes following spa treatment, MBT was selected as being the most diffuse and traditionally employed regimen across Europe, although the *thermal water* component, which is believed to be responsible of the therapeutic effect of spa therapy, is the base of all the three regimens clinically assessed. The MBT group was also selected for the proteomic study in order to avoid the *physical exercise bias* present in the other two groups. Blood samples from the patients enrolled in the study were collected by venipuncture, immediately centrifuged and the serum was stored at –30°C until analyzed. For the proteomic analysis, patients' sera from the MBT group were pooled in two different sample mixtures in order to have biological replicates. The two pools of sera were representative of the male:female ratio and age distribution of the entire cohort. Serum proteins (listed in the supplementary data, see Appendix) were profiled using a semi-quantitative method by the biotin-labeled-based protein array (RayBio® L-Series Human Antibody Array 1000), following the manufacturer's instructions. Once the glass slides were dried, laser fluorescence scanning was used to visualize signals. The images were captured using an Axon GenePix laser scanner and are available in the Appendix. After subtracting background signals and normalization to positive controls,

Table I - Chemical and physical characteristics of thermal water.

Bicarbonates	26.0 meq/L
Calcium	27.0 mg/L
Chlorides	530.0 mg/L
Iron	0.30 mg/L
Fluorides	10.1 mg/L
Magnesium	6.6 mg/L
Nitrates	1.4 mg/L
Nitrites	0.020 mg/L
Potassium	41.0 mg/L
Silica	55.6 mg/L
Sodium	1220.0 mg/L
Sulphate	56.0 mg/L
Phosphates	0.0 mg/L
Free carbon dioxide	115.0 mg/L
Electrical conductivity (at 20°C)	3380 micrS/cm
Oxidizability	0.8 mgO_2/L
pH	7.4 adimens
Fixed residue (at 180°)	2425 mg/L

Chemical and physical characteristics of the Sardara thermal water employed in this study (sodium bicarbonate alkaline water, 56.4°C).

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comparison of signal intensities between array images was employed to determine relative differences in expression levels of each protein between groups. According to the manufacturer's technical datasheet, any ≥2.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte was considered a measurable and significant difference in expression, provided that both sets of signals were well above background (mean background + 2 standard deviations, accuracy ≈95%). Proteome data analysis was performed using the Analysis Tool Software for RayBio® Human Biotin-Label Based Antibody Arrays, which automatically normalize signal intensities to the array's positive controls. The results are shown in terms of fold increase or decrease between the end of treatment (T2W) and the baseline.

For the *in silico* interactome analysis, STRING open-access software (27) was used to analyze multiple protein-protein interactions (https://string-db.org/). STRING software scores are indicators of confidence, i.e. how likely STRING judges an interaction to be true, given the available evidence. All scores rank from 0 to 1, with 1 being the highest possible confidence. A score of 0.5 would indicate that roughly every second interaction might be erroneous (*i.e.*, a false positive). Demographic and clinical data have been expressed as mean ±SD. The Wilcoxon test was used to compare baseline data with data obtained after treatment at 2 and 12 weeks. P values less than 0.05 were considered statistically significant. Statistical analysis has been performed with the GraphPad Prism software V 5.0.

RESULTS

In this study we investigated the effect of thermal water treatments in spa environment in patients with chronic back pain, focusing on changes induced in serum proteins by analyzing, before and after MBT, one thousand different serum proteins covering the principal physiological functions. The whole cohort of spa-treated axOA patients (46 subjects) showed a clear-cut benefit both after two weeks and after 12 weeks compared with baseline, while no improvements were observed in the axOA control group (20 subjects). To sum up, in the whole cohort of spa-treated axOA patients we found: VAS pain at baseline 6.2±1.6, T2W 3.4±1.3 and T12W 4.5±1.5 (baseline *vs* T2W p=0.0001, baseline *vs* T12W p=0.0001). Neck disability index at baseline was 62.1±10.4, T2W 37.3±13.6 and T12W 44.7±12.2 (baseline *vs* T2W p=0.0001, baseline *vs* T12W p=0.0001). Roland Morris index at baseline was 15.6±3.7, T2W 7.7±2.9 and T12W 10.9±4.0 (baseline *vs* T2W p=0.0001, baseline *vs* T12W p=0.0001). Detailed results in all axOA treatment groups and axOA controls are shown in Table II; because of the limited number of patients, inter-groups comparison was not performed.

As a parallel group, 11 patients with chronic back pain secondary to AS, with inactive inflammatory disease, were also treated with the HRS, similarly to group C. In detail: VAS pain at baseline was 6.3±2.2, T2W 3.8±2.7 and T12W 2.9±2.3 (baseline *vs* T2W p=0.021, baseline *vs* T12W p=0.005). BASMI at baseline was 5.3±2.6, T2W 3.2±2.0 and T12W 3.4±2.1 (baseline *vs* T2W p=0.005, baseline *vs* T12W p=0.014). BASDAI at baseline was 5.1±2.8, T2W 2.9±2.7 and time T12W 2.6±1.5 (baseline *vs* T2W p=0.005, baseline *vs* T12W p=0.001). BASFI at baseline was 4.0±1.6, T2W 2.6±1.6 and time T12W 3.0±1.3 (baseline vs T2W p=0.014, baseline *vs* T12W p=0.018). SF-36 PC at baseline was 34.7±9.2, T2W 39.2±12.3 and T12W 40.6±14.6 (baseline *vs* T2W p=ns, baseline *vs* T3M p=ns); SF-36 MC at baseline was 43.8±10.8, T2W 51.5±11.7 and T12W 54.8±12.0 (baseline *vs* T2W p=0.005, baseline *vs* T12W p=0.042).

In this study we applied a proteomic approach to evaluate the effects of MBT (avoiding the bias of physical exercise) on a large spectrum of serum proteins in patients with chronic back pain secondary to osteoarthritis. Profiling 1000 serum proteins (the full list of tested proteins is shown as a supplementary file, see Appendix) we found 13 serum proteins (listed in Table III with

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Table II - Changes in clinical data following spa treatments.

Treatment regimen	Domain/instrument	Baseline	2 Weeks	12 Weeks
Group A MBT	Vas PAIN	5.3±1.0	2.7±0.9 p=0.001	4.0±1.0 p=0.003
	Neck disability index	57.7±9.4	36.7±8.5 p=0.035	46.1±9.7 p=0.031
	Roland Morris	15.0±4.5	7.0±4.6 p=0.035	10.2±4.9 p=0.031
	SF-36 PC	37.2±4.9	46.8±3.0 p=0.005	42.0±3.6 p=0.002
	SF-36 MC	43.7±7.1	44.3±5.5 p=ns	41.3±5.8 p=ns
Group B MBT + HRS	Vas PAIN	5.5±1.3	3.0±1.4 p=0.001	3.8±1.6 p=0.003
	Neck disability index	66.7±7.6	40.1±16.7 p=0.014	44.2±17.3 p=0.007
	Roland Morris	14.2±2.4	6.6±2.6 p=0.014	9.0±2.4 p=0.014
	SF-36 PC	35.2±7.2	44.8±8.2 p=0.000	40.0±8.0 p=0.005
	SF-36 MC	41.5±7.3	43.5±8.5 p=0.021	42.9±7.3 p=ns
Group C HRS	Vas PAIN	7.4±1.5	4.1±1.1 p=0.000	5.4±1.3 p=0.000
	Neck disability index	60.7±13.0	34.7±14.5 p=0.015	44.2±8.0 p=0.001
	Roland Morris	17.0±3.9	8.9±2.5 p=0.003	12.7±4.0 p=0.006
	SF-36 PC	32.2±6.2	44.7±6.0 p=0.000	38.3±4.7 p=0.000
	SF-36 MC	41.3±8.0	41.6±8.5 p=ns	41.9±7.2 p=ns
Control group NO spa therapy	Vas PAIN	6.4±1.8	6.1±1.5 p=ns	
	Neck disability index	55.5±16.0	52.3±14.6 p=ns	
	Roland Morris	15.9±3.0	15.1±3.4 p=ns	
	SF-36 PC	45.0±6.0	41.9±5.5 p=ns	
	SF-36 MC	43.0±6.0	43.0±7.0 p=ns	

Changes in clinical data following spa treatments in the different axOA groups according to: VAS pain, Neck disability index, Roland Morris disability questionnaire, Short form healthy survey 36 items (SF-36) physical and mental components (PC and MC, respectively). Because of the limited number of patients, inter-groups comparison was not performed. Abbreviations: Mud bath therapy (MBT), Hydrotherapy rehabilitation scheme (HRS), axial osteoarthritis (axOA).

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details of their biological functions) that were greatly modulated (≥2.5-fold increase or ≤0.65 fold decrease) comparing baseline and the end of 2 weeks of MBT treatment in both biological replicates. The fold variation that was not measured concordantly in both biological replicates, was not considered valid and the relative proteins were excluded. In detail, the following serum proteins were found to be greatly increased after spa treatment: inhibin beta A subunit (INHBA), activin A receptor type 2B (ACVR2B), angiopoietin-1 (ANGPT1), beta-2-microglobulin (B2M), growth differentiation factor 10 (GDF10), C-X-C motif chemokine ligand 5 (CXCL5), fibroblast growth factor 2 (FGF2), fibroblast growth factor 12 (FGF12), oxidized low density lipoprotein receptor 1 (OLR1), matrix metallopeptidase 13 (MMP13). The following three proteins were greatly decreased: apolipoprotein C-III (Apoc3), interleukin 23 alpha subunit p19 (IL23A) and syndecan-1 (SDC1).

Apart from the possible specific role and functions of each single overexpressed or downregulated protein, which are summarized in Table III, we also underline the possible protein-protein interactions. As shown in Figure 1, the *in silico* analysis of interaction between multiple proteins showed some interesting and strong data-supported connections between 10 out of 13 proteins changed after MBT treatment (Figure 1A). Expanding the panoramic view to other secondary predicted interactions around these proteins, the network was amplified as a result of software input by a maximum of 5

proteins around each node (Figure 1B). To verify how likely STRING software judges an interaction to be true (given the available evidence), we reported in Table IV all rank of confidence scores assigned automatically by the software.

DISCUSSION AND CONCLUSIONS

The aim of the clinical part of this study was not to prove the efficacy of spa therapy in chronic back pain, which has been

Table III - List of serum proteins dysregulated after MBT treatment and their functions.

L-series array name	Full-name protein	Protein annotations	Fold
IL-23	Interleukin 23, alpha subunit p19 (IL23A)	Associates with IL12B to form the IL-23 interleukin, a heterodimeric cytokine which functions in innate and adaptive immunity. IL-23 induces inflammation and is involved in immune mediated inflammatory diseases.	0.4
Syndecan-1	Syndecan-1 (SDC1)	Cell surface proteoglycan that bears both heparan sulfate and chondroitin sulfate and that links the cytoskeleton to the interstitial matrix. Regulates exosome biogenesis in concert with SDCBP and PDCD6IP.	0.5
ApoC3	Apolipoprotein C-III (APOC3)	Component of triglyceride-rich very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL) in plasma. Plays a multifaceted role in triglyceride homeostasis.	0.6
BMP-3b / GDF-10	Growth differentiation factor 10 (GDF10)	Involved in osteogenesis and adipogenesis. Plays an inhibitory role in the process of osteoblast differentiation via SMAD2/3 pathway, and in the process of adipogenesis.	2.5
Activin A	Inhibin beta A subunit (INHBA)	Regulates a number of diverse functions such as hypothalamic and pituitary hormone secretions including follitropin.	2.7
FGF Basic	Fibroblast growth factor 2 (FGF2)	Regulation of cell survival, cell division, angiogenesis, cell differentiation and cell migration. Functions as potent mitogen *in vitro*.	2.7
Angiopoietin-1	Angiopoietin-1 (ANGPT1)	Regulation of angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading, reorganization of the actin cytoskeleton, but also maintenance of vascular quiescence.	3.2
FGF-12	Fibroblast growth factor 12 (FGF12)	Involved in nervous system development and function.	3.2
pro-MMP13	Matrix metallopeptidase 13 (MMP13)	Degradation of extracellular matrix proteins including fibrillar collagen, fibronectin, TNC and ACAN. Plays a role in wound healing, tissue remodeling, cartilage degradation, bone development, bone mineralization and ossification.	4.2
ENA-78	C-X-C motif chemokine ligand 5 (CXCL5)	Involved in neutrophil activation.	4.2
Activin RII A/B	Activin A receptor type 2B (ACVR2B)	Transduces the activin signal from the cell surface to the cytoplasm and thus regulates many physiological and pathological processes including wound healing, extracellular matrix production, immunosuppression and carcinogenesis.	4.2
LOX-1	Oxidized low density lipoprotein receptor 1 (OLR1)	Marker of atherosclerosis that induces vascular endothelial cell activation and dysfunction, resulting in pro-inflammatory responses, pro-oxidative conditions and apoptosis. Also involved in inflammatory process.	34.9
Beta 2M	Beta-2-microglobulin (B2M)	Component of the class I major histocompatibility complex (MHC). Involved in the presentation of peptide antigens to the immune system.	55.8

Results are expressed as mean of fold increase (≥2.5) or decrease (≤0.65) comparing baseline and 2-week therapy serum samples, concordantly in both biological replicates.

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already demonstrated in large cohorts of patients as previously summarized, but to explore different treatment regimens as a preliminary approach to future studies.

The results emerging from this study, although with the limitations due to the small number of patients enrolled in each group, allow several clinical observations to be drawn as to the beneficial effects of different spa treatment regimens in chronic back pain and provide novel data regarding protein changes following MBT, by means of a widespread proteomic approach. To sum up, we found a clear clinical benefit, as assessed by VAS pain, Neck disability index, Roland Morris disability questionnaire and SF-36 PC in axOA patients following spa intervention for all the three regimens employed. Because of the limited number of patients, inter-groups comparison was not performed.

In contrast, we did not find any clinical change in the control group of patients that continued with standard medication and did not receive spa or physiotherapy. It is also of note that the parallel group of AS patients, although direct comparison is clearly not possible, did experience a similar clinical benefit as in group C of the axOA patients (both groups received the HRS regimen), as assessed by means of VAS pain and disease specific BASMI, BASFI and BASDAI instruments, confirming previous reports (7). The overall analysis of the data emerging from the studies performed on spa therapy is difficult because they differ in terms of protocol, clinical profile of the recruited patients, differences in spa procedures, intensity and duration of treatment, as well as methods of assessments (18). Nevertheless, we can affirm that the avail-

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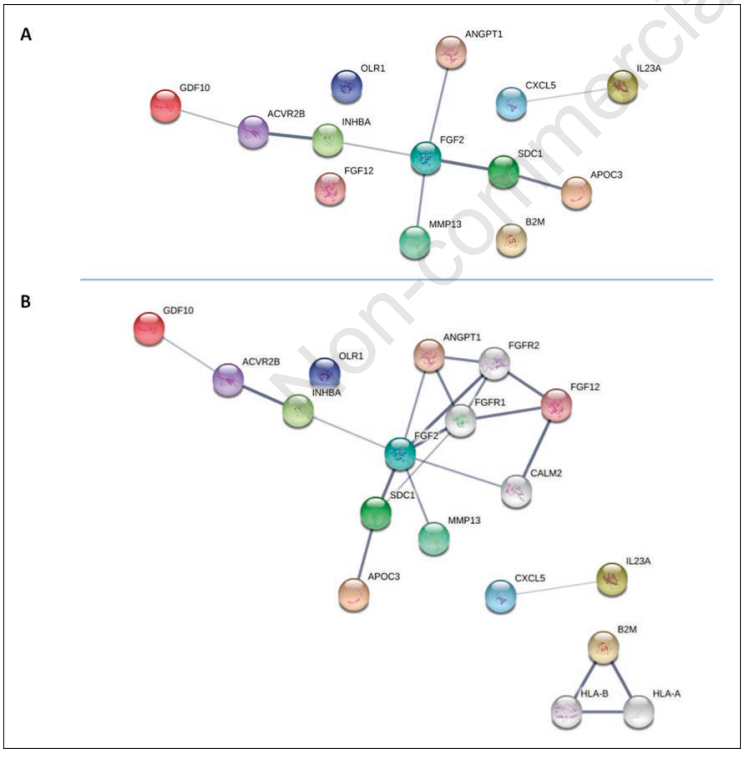

Figure 1 - *In silico* multiple-proteins interaction analysis (STRING software V 10.5). Network nodes represent proteins, edges represent protein-protein association (line thickness indicates the strength of data support). Panel A shows the first-grade association between the 13 proteins dysregulated after MBT treatment, in panel B the number of proteins is expanded to secondary interactions.

Table IV - Browse interactions in tabular form.

Node 1	Node 2	Score
ACVR2B	GDF10	0.52
ACVR2B	INHBA	0.99
ANGPT1	FGF2	0.70
APOC3	SDC1	0.90
CXCL5	IL23A	0.41
FGF2	ANGPT1	0.70
FGF2	INHBA	0.54
FGF2	MMP13	0.72
FGF2	SDC1	0.98
GDF10	ACVR2B	0.52
INHBA	ACVR2B	0.99
INHBA	FGF2	0.54
IL23A	CXCL5	0.41
MMP13	FGF2	0.72
SDC1	APOC3	0.90
SDC1	FGF2	0.98

In STRING software, each protein-protein interaction is annotated with one or more *scores*. These scores are indicators of confidence, all scores rank from 0 to 1, with 1 being the highest possible confidence. A score of 0.5 would indicate that roughly every second interaction might be erroneous (*i.e.*, a false positive).

able evidence in the scientific literature suggests a clear therapeutic benefit of spa treatments in several musculoskeletal diseases (29-32). In order to contribute to the understanding of the mechanisms responsible for these beneficial effects reported by the patients, we also investigated the changes induced by MBT in serum protein profile.

In our wide-spectrum protein analysis we obtained, by direct antigen-labeling technology (Biotin Label-based Antibody Array), a broad and panoramic view of protein expression. Using this semi-quantitative technique, up to 1000 target proteins were simultaneously detected, making this approach ideally suited for proteomic studies. We observed in both biological replicates increased levels (>2.5 fold) of: inhibin beta A subunit (INHBA), activin A receptor type 2B (ACVR2B), Angiopoietin-1 (ANGPT1), beta-2-microglobulin (B2M), growth differentiation factor 10 (BMP-3b/GDF10), C-X-C motif chemokine ligand 5 (CXCL5), fibroblast growth factor 2 (FGF2), fibroblast growth factor 12 (FGF12), oxidized low density lipoprotein receptor 1 (OLR1), matrix metallopeptidase 13 (MMP13). We observed that some increased proteins belongs to the same family or pathway: INHBA, ACVR2B and BMP-3b are members of TGF-Beta superfamily; CXCL5 and MMP13 participate in the IL-17 signaling pathway; FGF2 and FGF12 are proteins of FGF family. Among the three decreased proteins (≤0.65 fold) we underline the key role played by interleukin-23 in immune mediated diseases (30), and the role of apolipoprotein C-III (Apoc3) and syndecan-1 (SDC1) in triglyceride and interstitial matrix physiology.

Although it is not possible to speculate as to a specific role for each dysregulated protein in the beneficial effect of MBT, these results are extremely interesting because they show that MBT may induce changes in proteins involved in functions such as modulation of gene expression, cell differentiation, angiogenesis and tissue repair, acute and chronic inflammatory response. In this regard, it is of note that some of these proteins may also be linked by physiologic interactions, as shown by the *in silico* analysis of associations between multiple proteins in Figure 1. For a precise description of a protein's function, knowledge about its interactions with other proteins is clearly important. We have adopted the STRING software as a tool to derive functional associations, likely contributing to a common biological purpose. Interactions were derived from:

1) known experimental interactions obtained from scientific databases;
2) pathway knowledge from database;
3) text-miming to uncover links between proteins based on Medline search;
4) predicted interactions using genomic information (33, 34).

These data need to be interpreted in the context of all the data available in the literature, and represent a first approach to an *omic* strategy in the study of spa treatments. The wide-spectrum analysis, obtained from the semi-quantitative array that we employed, allows detection only of *great* changes, therefore small (but eventually important) physiological variations of other proteins have not been detected. In this regard, several studies have provided evidence for the protective role of spa treatments on cartilage and bone by means of a significant reduction in serum levels of IL-1, TNF-α (35), PGE2 and LTB4 (36), which are key cytokines in the joint inflammatory milieu and are also involved in osteoclast recruitment and bone reabsorption pathways. Moreover, it has been shown that MBT increase serum levels of osteocalcin (bone GLA protein, BGP), produced by osteoblasts, and bone alkaline phosphatase, both considered markers of bone metabolism (37). Several authors have also reported changes in markers of cartilage degradation and circulating levels of adiponectin, resistin and visfatin after MBT in patients with OA (38).

Taking all these data together, we may assume that the chemical properties of mineral water, together with mechanical and thermal effects (12), may explain, at least

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in part, the clinical benefits and serum protein changes observed in our cohort of patients, as well as in real life rheumatic patients (39). In the attempt to clarify the mechanism of action of spa treatments we should also consider the pain control theory (40, 41), the result of hydrostatic pressure on blood circulation as well as the impact on muscle spasm of the heat, all mechanisms which may mediate pain reduction and function improvement (42, 43). Hydrotherapy and balneotherapy have been also reported to exert beneficial effects on mood, anxiety and depression, therefore contributing to the amelioration of pain and general quality of life scores (44), although our data do not show an improvement in SF-36 MC index.

Limitations of the study

The main objective of the study was to evaluate protein changes following MBT and therefore we acknowledge that the number of patients enrolled is limited for a pure clinical study. The cohort was analyzed according to a single-blind method and therefore the patients were aware of their treatment regime. The assessment was also based on self-reported questionnaires and therefore the placebo effects could not be weighted. Furthermore, the wide-spectrum analysis by a semi-quantitative technique was not able to detect small changes in proteins which may have biological importance.

To sum up, the data emerging from this study, although with the limitations previously listed, confirm that spa therapy appears to be beneficial for chronic back pain, probably by means of different mechanisms, and to induce changes in proteins involved in functions such as gene expression modulation, cell differentiation, angiogenesis, tissue repair, acute and chronic inflammatory response.

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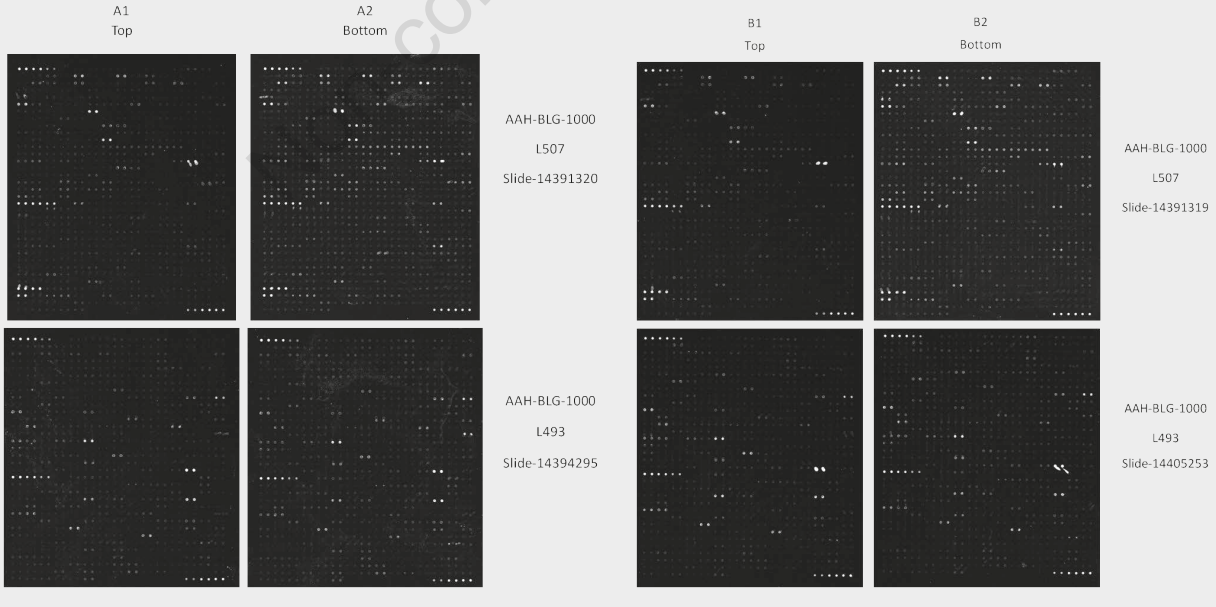
APPENDIX



RayBio® L-Series Human Antibody Array 1000 probed with serum samples. The images of the glasses were captured using an Axon GenePix laser scanner. The strong signals in row 20 and the upper left and lower right corners of each array are Positive Controls, which can be used to identify the orientation and help normalize the results between arrays.

Full list of proteins tested with "Human L1000 Array, Glass Slide" (RayBiotech): Target Name

11b-HSD1, 2B4, 4-1BB, 6Ckine, A1BG, A2M, ABL1, ACE, ACE-2, ACK1, ACPP, ACTH, Activin A, Activin B, Activin C, Activin RIA / ALK-2, Activin RIB / ALK-4, Activin RII A/B, Activin RIIA, ADAM-9, ADAMTS-1, ADAMTS-10, ADAMTS-13, ADAMTS-15, ADAMTS-17, ADAMTS-18, ADAMTS-19, ADAMTS-4 , ADAMTS-5, ADAMTS-L2, Adiponectin / Acrp30, Adipsin, Afamin, AFP, AgRP, ALBUMIN, ALCAM, Aldolase A, Aldolase B, Aldolase C, ALK, Alpha 1 AG, Alpha 1 Microglobulin, Alpha Lactalbumin, ALPP, AMICA, AMPKa1, Amylin, Angiogenin, Angiopoietin-1, Angiopoietin-2, Angiopoietin-4, Angiopoietin-like 1, Angiopoietin-like 2, Angiopoietin-like Factor, Angiostatin, ANGPTL3, ANGPTL4, Annexin A7, APC, APCS, Apelin, Apex1, APJ, APN, ApoA1, ApoA2, ApoA4, ApoB, ApoB100, ApoC1, ApoC2, ApoC3, ApoD, ApoE, ApoE3, ApoH, ApoM, APP, APRIL, AR (Amphiregulin), Artemin, ASPH, Attractin, Axl, B3GNT1, B7-1 /CD80, BACE-1, BAF57, BAFF, BAFF R / TNFRSF13C, BAI-1, bax, BCAM, BCMA / TNFRSF17, BD-1, BDNF, Beta 2M, Beta Defensin 4, Beta IG-H3, beta-Catenin, beta-NGF, Biglycan, BIK, BLAME, BLC / BCA-1 / CXCL13, BMP-15, BMP-2, BMP-3, BMP-3b / GDF-10, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMPR-IA / ALK-3, BMPR-IB / ALK-6, BMPR-II, BMX, BNIP2, BNP, BTC, Btk, C2, C3a, C5/C5a, C7, C8B, C9, CA125, CA15-3, CA19-9, CA9, Cadherin-13, Calbindin, Calbindin D, Calcitonin, Calreticulin, Calsyntenin-1, Cardiotrophin-1 / CT-1, CART, Caspase-3, Caspase-8, Cathepsin B, Cathepsin D, Cathepsin L, Cathepsin S, CBP, CCK, CCL14 / HCC-1 / HCC-3, CCL28 / VIC, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CD 163, CD14, CD200, CD23, CD24, CD27 / TNFRSF7, CD30 / TNFRSF8, CD30 Ligand / TNFSF8, CD36, CD38, CD40 / TNFRSF5, CD40 Ligand / TNFSF5 /CD154, CD44, CD45, CD46, CD47, CD55, CD59, CD61, CD71, CD74, CD79 alpha, CD90, CD97, CEA, CEACAM-1, Cerberus 1, Ceruloplasmin, CFHR2, Chem R23, Chemerin, CHI3L1, Chordin-Like 1, Chordin-Like 2, Chromogranin A, Chymase, cIAP-2, Ck beta 8-1, CK-MB, Claudin-3, Claudin-4, CLC, CLEC3B, Clusterin, CNDP1, CNTF R alpha, CNTF, Coagulation Factor III / Tissue Factor, Coagulation Factor X/Xa, COCO, Complement factor H, Contactin-1, Contactin-2, Corticosteroid-binding globulin, COX-2, C-peptide, CPN2, Creatinine, CRIM 1, Cripto-1, CRP, CRTAM, CRTH-2, Cryptic, CSH1, Csk, CTACK / CCL27, CTGF / CCN2, CTLA-4 /CD152, cTnT / Troponin T, CutA, CV-2 / Crossveinless-2, CXCL14 / BRAK, CXCL16, CXCR1 / IL-8 RA, CXCR2 / IL-8 RB, CXCR3, CXCR4 (fusin), CXCR5 /BLR-1, CXCR6, Cyclin D1, Cystatin A, Cystatin B, Cystatin C, Cytochrome C, Cytokeratin 8, Cytokeratin18, Cytokeratin19, D6, DAN, DANCE, DBI, DCBLD2, DcR3 / TNFRSF6B, D-Dimer, Decorin, DEFA1/3, Defensin, Desmin, Dkk-1, Dkk-3, Dkk-4, DLL1, DLL4, DMP-1, DPPIV, DR3 / TNFRSF25, DR6 / TNFRSF21, Dtk, E-Cadherin, EDA-A2, EDAR, EDG-1, EGF, EGF R / ErbB1, EG-VEGF / PK1, EMAP-II, ENA-78, Endocan, Endoglin / CD105, Endorphin Beta, Endostatin, Endothelin, Endothelin Receptor A, Enolase 2, ENPP2, EN-RAGE, Eotaxin / CCL11, Eotaxin-2 / MPIF-2, Eotaxin-3 / CCL26, EpCAM, EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphB1, EphB2, EphB3, EphB4, EphB6, Epiregulin, ErbB2, ErbB3, ErbB4, ERRa, Erythropoietin R, Erythropoietin, ESAM, E-Selectin, EV15L, EXTL2, FABP1, FABP2, FABP3, FABP4, Factor XIII A, Factor XIII B, FADD, FAK, FAM3B, FAP, Fas / TNFRSF6, Fas Ligand, Fc RIIB/C, Fen 1, FER, Ferritin, Fetuin A, Fetuin B, FGF Basic, FGF R3, FGF R4, FGF R5, FGF-10 / KGF-2, FGF-11, FGF-12, FGF-13 1B, FGF-16, FGF-17, FGF-18, FGF-19, FGF-20, FGF-21, FGF-23, FGF-4, FGF-5, FGF-6, FGF-7 / KGF, FGF-8, FGF-9, FGF-BP, FGFR1, FGFR1 alpha, FGFR2, Fibrinogen, Fibrinopeptide A, Fibronectin, Ficolin-3, FIH, FLRG, Flt-3 Ligand, Follistatin, Follistatin-like 1, FOLR1, FOXN3, FoxO1, FoxP3, Fractalkine, Frizzled-1, Frizzled-3, Frizzled-4, Frizzled-5, Frizzled-6, Frizzled-7, FRK, FSH, Furin, Fyn," GADD45A, Galanin, Galectin-1, Galectin-3, Galectin-3BP, Galectin-7, gamma-Thrombin, Gas1, GASP-1 / WFIKKNRP, GASP-2 / WFIKKN, Gastrin, GATA-3, GATA-4, GCP-2 / CXCL6, GCSF, G-CSF R / CD 114, GDF1, GDF11, GDF-15, GDF3, GDF5, GDF8, GDF9, GDNF, Gelsolin, GFR alpha-1, GFR alpha-2, GFR alpha-3, GFR alpha-4, Ghrelin, GITR / TNFRF18, GITR Ligand / TNFSF18, GLO-1, GLP-1, Glucagon, Glut1, Glut2, Glut3, Glut5, Glypican 3, Glypican 5, GM-CSF, GM-CSF R alpha, GMNN, GPBB, GPI, GPR-39, GPX1, GPX3, Granzyme A, Grb2, GREMLIN, GRO, GRO-a, Growth Hormone (GH), Growth Hormone R (GHR), GRP, GRP75, GRP78, GSR, GST, HADHA, HAI-1, HAI-2, Haptoglobin, HB-EGF, HCC-4 / CCL16, hCG alpha, hCGb, Hck, HCR / CRAM-A/B, HE4, Hemopexin, Hepassocin, Hepcidin, HGF, HGFR, HOXA10, HRG-alpha, HRG-beta 1, HSP10, HSP20, HSP27, HSP32, HSP40, HSP60, HSP70, HSP90, HSPA8, HTRA2, HVEM / TNFRSF14, I-309, IBSP, ICAM-1, ICAM-2, ICAM-3 (CD50), ICAM-5, IFN-alpha / beta R1, IFN-alpha / beta R2, IFN-beta, IFN-gamma, IFN-gamma R1, IGF2BP1, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, IGFBP-rp1 / IGFBP-7, IGF-I, IGF-I SR, IGF-II, IGF-II R, IL-1 alpha, IL-1 beta, IL-1 F10 / IL-1HY2, IL-1 F5 / FIL1delta, IL-1 F6 / FIL1 epsilon, IL-1 F7 / FIL1 zeta, IL-1 F8 / FIL1 eta, IL-1 F9 / IL-1 H1, IL-1 R3 / IL-1 R AcP, IL-1 R4 /ST2, IL-1 R6 / IL-1 Rrp2, IL-1 R8, IL-1 R9, IL-1 ra, IL-1 sRI, IL-1 sRII, IL-10, IL-10 R alpha, IL-10 R beta, IL-11, IL-12 p40, IL-12 p70, IL-12 R beta 1, IL-12 R beta 2, IL-13, IL-13 R alpha 1, IL-13 R alpha 2, IL-15, IL-15 R alpha, IL-16, IL-17, IL-17B, IL-17B R, IL-17C, IL-17D, IL-17E, IL-17F, IL-17R, IL-17RC, IL-17RD, IL-18 BPa, IL-18 R alpha /IL-1 R5, IL-18 R beta /AcPL, IL-19, IL-2, IL-2 R alpha, IL-2 R beta /CD122, IL-2 R gamma, IL-20, IL-20 R alpha, IL-20 R beta, IL-21, IL-21 R, IL-22, IL-22 BP, IL-22 R, IL-23, IL-23 R, IL-23p19, IL-24, IL-26, IL-27, IL-28A, IL-29, IL-3, IL-3 R alpha, IL-31, IL-31 RA, IL-33, IL-34, IL36RN, IL-4, IL-4 R, IL-5, IL-5 R alpha, IL-6, IL-6 R, IL-7, IL-7 R alpha, IL-8, IL-9 , INSL3, INSRR, Insulin, Insulin R, Insulysin / IDE, Integrin alpha V, IP-10, I-TAC / CXCL11, Itk, ITM2B, Kallikrein 10, Kallikrein 11, Kallikrein 14, Kallikrein 2, Kallikrein 5, Kallikrein 6, Kallikrein 7, Kallikrein 8, KCC3, KCTD10, KIF3B, Kininostatin / kininogen, KLF4, Kremen-1, Kremen-2, LAG-3, Latent TGF-beta bp1, Layilin, LBP, Lck, LDL R, LECT2, Lefty - A, Legumain,

Leptin (OB), Leptin R, LFA-1 alpha, LH, LIF R alpha, LIF, LIGHT / TNFSF14, LIMPII, LIN41, Lipocalin-1, Livin, LOX-1, LPS, LRG1, LRP-1, LRP-6, L-Selectin (CD62L), LTF, LTK, Luciferase, Lumican, Lymphotactin / XCL1, Lymphotoxin beta / TNFSF3, Lymphotoxin beta R / TNFRSF3, Lyn, LYRIC, LYVE-1, LZTS1, MAC-1, Mammaglobin A, Marapsin, MATK, MBL, MBL-2, MCP-1, MCP-2, MCP-3, MCP-4 / CCL13, M-CSF, M-CSF R, MDC, Mer, Mesothelin, MFG-E8, MFRP, MICB, Midkine, MIF, MIG, MINA, MIP 2, MIP-1a, MIP-1b, MIP-1d, MIP-3 alpha, MIP-3 beta, MMP-1, MMP-10, MMP-11 /Stromelysin-3, MMP-12, MMP-13, MMP-14, MMP-15, MMP-16 / MT3-MMP, MMP-19, MMP-2, MMP-20, MMP-24 / MT5-MMP, MMP-25 / MT6-MMP, MMP-3, MMP-7, MMP-8, MMP-9, MSHa, MSP alpha Chain, MSP beta-chain, MTUS1, Musk, Myoglobin, NAIP, Nanog, NAP-2, NCAM-1 / CD56, NELL2, NEP, Nesfatin, Nestin, NET1, Netrin G2, Netrin-4, Neuritin, NeuroD1, Neurokinin-A, Neuropeptide Y, Neuropilin-2, Neurturin, NF1, NGF R, NM23-H1/H2, Notch-1, NOV / CCN3, NPTX1, NPTXR, NR3C3, NRG1 Isoform GGF2, NRG1-alpha / HRG1-alpha, NRG1-beta1 / HRG1-beta1, NRG2, NRG3, NT-3, NT-4, Ntn1, OCT3/4, Omentin, Orexin A, Orexin B, OSM, Osteoactivin / GPNMB, Osteocalcin, Osteocrin, Osteopontin, Osteoprotegerin / TNFRSF11B, OX40, OX40 Ligand / TNFSF4, p21, p27, p53, PAI-1, PAK7, Pancreastatin, Pancreatic Polypeptide, Pappalysin-1, PARC / CCL18, PARK7, P-Cadherin, PCAF, PD-1, PD-ECGF, PDGF R alpha, PDGF R beta, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-C, PDGF-D, PDX-1, PECAM-1 /CD31, PEDF , Pentraxin3 / TSG-14, PEPSINOGEN I, PEPSINOGEN II, Peroxiredoxin 6 (Prdx6), Persephin, PF4 / CXCL4, PGRP-S, PI 16, PI 3Kinase p85 beta, PIM2, PKM2, Plasminogen, PlGF, PLUNC, Podocalyxin, POMC, PON1, PON2, PPARg2, PPP2R5C, Pref-1, Presenilin 1, Presenilin 2, Pro-BDNF, Procalcitonin, Pro-Cathepsin B, Progesterone, pro-Glucagon, Progranulin, Prohibitin, Prolactin, Pro-MMP-13, Pro-MMP-7, Pro-MMP-9, ProSAAS, Prostasin, Protein p65, PSA-Free, PSA-total, P-selectin, PSP, PTH, PTHLP, PTN, PTPRD, PYK2, PYY, RAGE, RANK / TNFRSF11A, RANTES, Ras, RBP4, RECK, RELM alpha, RELM beta, RELT / TNFRSF19L, Resistin, RET, RIP1, ROBO4, ROCK1, ROCK2, ROR1, ROR2, ROS, RYK, S100 A8/A9, S100A10, S100A4, S100A6, S100A8, S-100b, SAA, SART1, SART3, SCF, SCF R /CD117, SCG3, SDF-1 / CXCL12, Selenoprotein P, SEMA3A, Serotonin, Serpin A1, Serpin A12, Serpin A3, Serpin A4, Serpin A5, Serpin A8, Serpin A9, Serpin B5, Serpin D1, Serpin I1, SERTAD2, sFRP-1, sFRP-3, sFRP-4, sgp130, SHBG, SIGIRR, Siglec-5/CD170, Siglec-9, SLPI, SMAC, Smad 1, Smad 4, Smad 5, Smad 7, Smad 8, SNCG, Soggy-1, Somatotropin, Sonic Hedgehog (Shh N-terminal), SOST, SOX17, SOX2, SPARC, SPARCL1, Spinesin, SPINK1, SRMS, SSEA-1, SSEA-4, SSTR2, SSTR5, Survivin, SYK, Syndecan-1, Syndecan-3, TACE, TACI / TNFRSF13B, TAF4, Tarc, TCCR / WSX-1, Tec, TECK / CCL25, TFF1, TFF3, TFPI, TGF-alpha, TGF-beta 1, TGF-beta 2, TGF-beta 3, TGF-beta 5, TGF-beta RI / ALK-5, TGF-beta RII, TGF-beta RIII, Thrombin, Thrombomodulin, Thrombopoietin (TPO), Thrombospondin-1, Thrombospondin-2, Thrombospondin-4, Thymidine Kinase-1, Thymopoietin, Thyroglobulin, Thyroid Peroxidase (TPX), Tie-1, Tie-2, TIM-1, TIMP-1, TIMP-2, TIMP-3, TIMP-4, TL1A / TNFSF15, TLR1, TLR2, TLR3, TLR4, TMEFF1 / Tomoregulin-1, TMEFF2, TNF RI / TNFRSF1A, TNF RII / TNFRSF1B, TNF-alpha, TNF-beta, TNK1, TOPORS, TPA, TPM1, TRA-1-60, TRA-1-81, TRADD, TRAIL R1 / DR4 / TNFRSF10A, TRAIL R2 / DR5 / TNFRSF10B, TRAIL R3 / TNFRSF10C, TRAIL R4 / TNFRSF10D, TRAIL / TNFSF10, TRANCE, Transferrin, Trappin-2, TREM-1, TRKB, Troponin C, Troponin I, TROY / TNFRSF19, TRPC1, TRPC6, TRPM7, Trypsin 1, TSG-6, TSH, TSLP, TSLP, TWEAK / TNFSF12, TWEAK R / TNFRSF12, TXK, Tyk2, TYRO10, Ubiquitin+1, uPA, uPAR, Uromodulin, Vasopressin, Vasorin, VCAM-1 (CD106), VDUP-1, VE-Cadherin, VEGF, VEGF R1, VEGF R2 (KDR), VEGF R3, VEGF-B, VEGF-C, VEGF-D, VEGI / TNFSF15, VGF, VIP Receptor 2, Visfatin, Vitamin D Receptor, Vitamin D-BP, Vitamin K-dependent protein S, Vitronectin, VWF, WIF-1, Wilms Tumor 1, WISP-1 / CCN4, XEDAR, XIAP, ZAG, ZAP70

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